The role of the glutathione S-transferases in resistance to cytotoxic compounds

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PhD

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1990

I dedicate this Thesis to the fond memory of Aunty V. who always was, and will continue to be a source of inspiration to me.

DECLARATION

I hereby declare that:-

(a) This thesis has been composed by myself and

(b) The work contained herein is my own, unless otherwise indicated.

ABSTRACTS AND PRESENTATIONS ARISING FROM RESEARCH

Wareing, C.J., Lewis, A.D., Hayes, J.D. and Wolf, C.R. Sensitivity of Tumour cell lines with differing Glutathione S-transferase profiles to cytoxic compounds. Oral presentation to the Northern Drug Resistance Group. Glasgow, May. 1987.

Wareing, C.J., Hayes, J.D. and Wolf, C.R. (1988) Overexpression of Glutathione S-transferase subunits in a cell line resistant to 1-chloro-2,4-dinitrobenzene. Oral Presentation and Abstract. *Proceedings from the British Association for Cancer Research and the British Association for Cancer Physicians.*

Wareing, C.J., Hayes, J.D. and Wolf, C. R. (1988) Dramatic Overexpression of Acidic and Basic Glutathione S-transferase subunits in a Human Lung Tumour Cell Line made Resistant to 1-chloro-2,4-dinitrobenzene. Poster Presentation and Abstract.*Proceedings from the 79th Annual Meeting of the American Association for Cancer Research.*

Wareing, C.J., Hayes, J.D. and Wolf, C. R. Dramatic Overexpression of Acidic and Basic Glutathione S-transferase subunits in a Human Lung Tumour Cell Line made Resistant to 1-chloro-2,4-dinitrobenzene. Poster Presentation at the 11th European Workshop on Drug Metabolism. Konstanz, West Germany, 1988.

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Wareing, C.J., Hayes, J.D. and Wolf, C.R. Relationship of the Stability of Overexpressed Glutathione S-transferase Subunits in a CDNB Resistant Lung Tumour Cell Line to CDNB Resistance. Oral Presentation. Northern Drug Resistance Group Meeting, Manchester, March, 1989.

PUBLICATIONS ARISING FROM RESEARCH

Lewis, A.D., Forrester, L.M., Hayes, J.D., Wareing, C.J., Carmichael, J., Harris, A.L., Mooghen, M. and Wolf C.R. (1989) Glutathione S-transferase isoenzymes in human tumours and tumour derived lines *Br. J. Cancer* 60 : 327-331.

Holmes, J., Wareing, C.J., Jacobs, A., Hayes, J.D., Padua, R.A. and Wolf, C.R. (1990) Glutathione S-transferase pi expression in leukaemia : a comparative analysis with mdr-1 data. *Br. J. Cancer* 62 : 209-212.

Wolf, C.R., Wareing, C.J., Black, S.M. and Hayes, J.D.(1990) In : Glutathione S-transferases and Drug Resistance. (Hayes, J.D., Pickett, C.B. and Mantle, T.J. Editors.) Taylor and Francis,London. 295-307.

ACKNOWLEDGEMENTS

There are many people who have helped me enormously over the last four years and I would like to thank them all (in no particular order) as follows. A big thankyou to John Hayes for all his advice, encouragement, enthusiasm and expertise and for his help in the preparation of this Thesis. Also thankyou to Roland Wolf for allowing me to carry out this research in his laboratory where I received expert technical and scientific advice . I would like to say a special thankyou to Alex Lewis, Angela Scott and Julie Moss for their patience with me. A special thankyou also to Roland for his advice and encouragement , the last four years certainly have been an experience ! Seriously though Roland, thanks for everything.

I would also like to thank the staff of the ICRF Laboratory of Molecular Pharmacolgy for their help and friendship as well as the staff of the University of Edinburgh Department of Clinical Chemistry for their help. A very big thankyou must also go to Anne Ward for typing this Thesis for me, your time and effort have been very much appreciated Anne. Thanks also to Mr. Sandy Bruce for his help with the art work in this Thesis.

Last but most certainly not least, I would like to say a big thankyou to all my family and friends who have been so supportive during the last four years. A special thankyou to Mum and Dad for all their love and encoragement without which I would never have made it this far.I would like to finish by expressing my gratitude to Steven for enduring the last few months and for all his help, support and confidence in me.

ABBREVIATIONS

GSH - Reduced Glutathione

GST - Glutathione S-transferase

CDNB - 1-Chloro-2,4-dinitrobenzene

CHP - Cumene hydroperoxide

Adr - Adriamycin

MDR - Multidrug resistance

SDS - Sodium dodecyl sulphate

AFB_1 - Aflatoxin B_1

PBS - Phosphate buffered saline

hplc - High pressure liquid chromotography

BCNU - 1,3-Bis(2-Chloroethyl)-1-nitrosourea

CCNU - 1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea

Methyl CCNU - Methyl-1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea

DNA - Deoxyribonucleic acid

RNA - Ribonucleic acid

ATP - Adenosine triphosphate

CHO - Chinese Hamster Ovary

TRIS - Tris (hydroxy methyl) methylamide

cDNA - Copy deoxyribonucleic acid

mRNA - Messenger ribonucleic acid

EDTA - Ethylenediaminetetra-acetic acid

MRC - Medical Research Council

DMSO - Dimethyl sulphoxide

MTT - [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

rpm - Revolutions per minute

DTT - Dithiothreitol

CDTA - Trans-1,2-Diaminocyclohexane - NNN'N'-tetra acetic acid

TBST	**-**	**Tris buffered saline, tween**
IgG	**-**	**Immunoglobulin G**
NaPPi	**-**	**Sodium pyrophosphate**
BSA	**-**	**Bovine serum albumin**
PVP	**-**	**Polyvinyl-pyrrolidone**
μCi	**-**	**Microcuries**
dH_2O	**-**	**Distilled water**
dCTP	**-**	**Deoxy cytosine triphosphate**
dGTP	**-**	**Deoxyguanosine triphosphate**
dATP	**-**	**Deoxy adenosine triphosphate**
dTTP	**-**	**Deoxy thymidine triphosphate**
O.D	**-**	**Optical density**
FCS	**-**	**Foetal calf serum**
HSP	**-**	**Heat shock protein.**
MOPS	**-**	**3-[N-morpholino]-propan sulphonic acid**
PAGE	**-**	**polyacrylamide gel electrophoresis**

ABSTRACT

The role that glutathione S-transferases (GST) play in the protection of tumour cells from cytotoxic insult was investigated. The possibility that GSTs may be involved in a stress-response was also explored.

Derived drug-resistant tumour cell lines as well as showing various changes commonly associated with drug resistance, also show overexpression of certain GSTs. In the majority of drug resistant tumour cell models however, the ability of the drugs against which resistance is seen to act as GST substrates has not been demonstrated. For this reason, the known GST substrate, 1-chloro-2,4-dinitrobenzene (CDNB) was used to generate a tumour cell line resistant to this compound. The resultant CDNB resistant lung tumour cell line ($CDNB^r$) exhibited marked overexpression of both alpha and pi class GST subunits. This cell line was also resistant to cumene hydroperoxide, a substrate for alpha class GST. However, no resistance to any of the anticancer drugs studied, was observed. The results demonstrate that the GSTs can protect cells against cytotoxic insult from certain alkylating agents and hydroperoxides and it is possible that the GSTs are involved in some sort of chemical stress response. However, in the CDNB resistant lung tumour cell line, GSTs do not appear to provide protection against a broad spectrum of anticancer drugs.

The role of the human class pi enzyme in the resistance of human leukaemia cells to antitumour agents was also investigated. The results of this show that in certain instances, the pi class enzyme may be involved in resistance mechanisms to a number of drugs including chlorambucil. This work however, highlighted the multifactoral nature of drug resistance.

In conclusion, although the GSTs certainly appear to have the capacity to protect cells from cytotoxic compounds, it is evident that they are not the sole factor which determines the response of tumour cells to drug treatment.

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CHAPTER 2 MATERIALS AND METHODS

CHAPTER 1

INTRODUCTION

1.1.0 RESISTANCE TO ANTICANCER DRUGS

One in five people dies from cancer. Death is a result of metastases in two thirds of these cases and progression of the local disease in the remaining one third (Harris & Hickson, 1988). The major obstacle in the effective treatment of these cancers is the resistance of tumour cells to antineoplastic drugs. Resistance can either be intrinsic, exemplified by tumours that are refractory to treatment, or is acquired during chemotherapy regimens, exemplified by tumours that initially respond to anticancer drug therapy but which relapse subsequently and become unresponsive to treatment.

Antitumour agents have varied mechanisms of action and cancer cells develop several ways of circumventing the toxic effects of drugs, which results in drug resistance. In order to improve treatment strategies, several approaches are being taken, the most important of which is the elucidation of the processes underlying the drug resistance phenomenon.

Resistance to anticancer drugs can fall into two catergories. Cancer cells may be resistant to or develop resistance to just one particular agent, as in the case of the antimetabolite anticancer drugs such as methotrexate, discussed below. Alternatively, cancer cells are resistant to or develop resistance to a range of structurally and functionally unrelated drugs. This is termed cross-resistance and different tumour types display a range of cross-resistance patterns. Multidrug resistance (MDR)

describes the phenomenon whereby cells display cross-resistance patterns to a group of anticancer drugs that derive from natural products. This type of resistance is commonly associated with one particular resistance mechanism involving a membrane glycoprotein termed P-glycoprotein. This is dicussed in more detail in section 1.1.4. Mechanisms of resistance to other anticancer drugs not derived from natural products are less well understood.

In establishing, more comprehensively, the mechanisms of resistance to antineoplastic agents, several therapeutic applications are envisaged. For example, if the proteins that confer resistance are identified, drug regimens may be developed to inhibit the activity of such proteins, thereby possibly overcoming the resistance. Novel anticancer drugs may also be developed that are not in the spectrum of agents commonly associated with drug resistance. Before discussing the factors involved in the drug resistance phenomenon that are already under investigation, the drugs used in current chemotherapy and their sites and mode of action will be reviewed.

1.1.1 Sites and mechanisms of action of the anticancer drugs

The drugs used in modern day chemotherapy regimens are classified as follows (Farmer, 1985):

1. Alkylating agents
2. Antimetabolites
3. Vinca alkaloids
4. Antimitotic antibiotics
5. Miscellaneous compounds

For several of the anticancer drugs, the mode of action and the biochemical basis of their toxicity is unknown and may occur via several mechanisms. For this reason it may not be possible to assign certain drugs using the above classification. However, until further information is available, it is a convenient way of categorising the drugs.

(a) The Alkylating Agents

Examples of the drugs in this group are the nitrogen mustards, aziridines, dimethylsulphonates, nitrosoureas and the epoxides. The drugs have been designated alkylating agents because they all contain at least one alkyl moiety that is able to bond covalently to other molecules (Hirono, 1961; Connors, 1966). A simple representation of this reaction is shown below:

$$RCH_2 - X \quad + \quad Y \text{ -----> } RCH_2 - Y + X$$

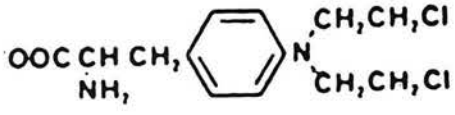
Alkylating agents in cancer chemotherapy, form covalent bonds with a number of biologically active macromolecules rendering them inactive. These macromolecules include DNA, RNA, proteins, amino acids and cell membrane constituents. The consequences of the covalent binding of alkylating agents to these molecules are DNA modification including formation of cross-links, inactivation of essential metabolic enzymes and membrane damage .

The nitrogen alkylating agents include nitrogen mustard, cyclophosphamide, melphalan and chlorambucil (Fig. 1.1). These compounds contain two potentially active alkyl groups.

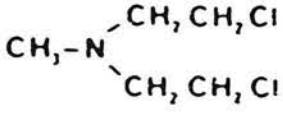
Cyclophosphamide undergoes enzymatic hydroxylation in the liver, resulting in the formation of 4-hydroxycyclophosphamide which can spontaneously convert to aldophosphamide. Non-enzymatic cleavage of this latter molecule produces acrolein and phosphoramide mustard. The phosphoramide mustard, the aldophosphamide and the 4-hydroxycyclophosphamide are all toxic, but it is thought to be the phosphoramide mustard which is responsible for the antineoplastic effect of cyclophosphamide (Freidman et al, 1979; Sladek 1987).

The nitrosourea alkylating agents are used widely in clinical practise. The three most important members of this group are BCNU, CCNU and methyl-CCNU. The alkylating activity of these drugs is thought to be their major cytotoxic action. In addition, their metabolites are able to inhibit the enzyme DNA polymerase and prevent the repair of DNA strand breaks and RNA synthesis (Gombar et al, 1981; Kohn 1977; Tew et al, 1981).

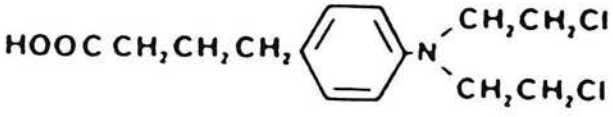
Figure 1.1 Examples of anticancer drugs in the group of compounds classed as alkylating agents.



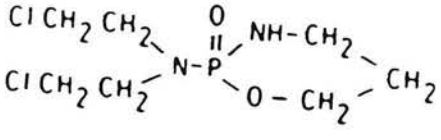
Melphalan



Nitrogen mustard



Chlorambucil



Cyclophosphamide

(b) **The Antimetabolites**

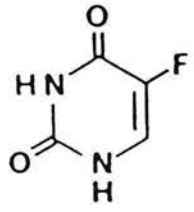
Before cells divide, nucleic acids, proteins and other cellular macromolecules are synthesised. Before this can occur, the precursor metabolites of the cellular constituents must also be synthesised. The antimetabolic neoplastic agents are very similar in structure to various precursor metabolites in nucleic acid biosynthesis pathways and are either incorporated into new nuclear material or combine with enzymes involved in precursor metabolism in an irreversible fashion, thus inhibiting their biological activity. As a result, either the new nuclear material will be unable to direct protein synthesis or enzyme inhibition will halt new nucleic acid biosynthesis. This breakdown in synthesis or functional integrity of these essential molecules will prevent further cell division.

The drug methotrexate is a folic acid antagonist. Tetrahydrofolates, which are essential cofactors in the synthesis of both purine and pyrimidine bases are formed by the enzymatic reduction of folic acid. It is the enzyme dihydrofolate reductase which converts folic acid to tetrahydrofolic acid which then goes on to form a number of tetrahydrofolates. Methotrexate has an affinity for dihydrofolate reductase and binds to it irreversibly thus blocking purine and pyrimidine synthesis (Farmer , 1985).

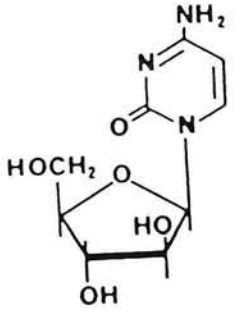
The antimetabolite 5-fluorouracil (Fig. 1.2) inhibits the enzyme thymidilate synthetase which catalyses the conversion of deoxyuridilic acid to thymidilic acid. This is an essential step in pyrimidine synthesis, and consequently, 5-fluorouracil prevents the formation of thymine and cytosine thereby arresting production of new DNA. This drug also affects RNA synthesis. Normal RNA contains the base uracil in place of thymine and as 5-fluorouracil is similar in structure to uracil, it becomes incorporated into RNA and halts protein transcription. It is not clear which of the two mechanisms detailed above is most important in the cytotoxic effect of this drug (Farmer ,1985).

Another drug which is classed as an antimetabolite is cytosine arabinoside (Fig. 1.2). This compound is an analogue of deoxycytidine and has a number of effects on DNA formation. It is known to inactivate DNA polymerase, thereby inhibiting both

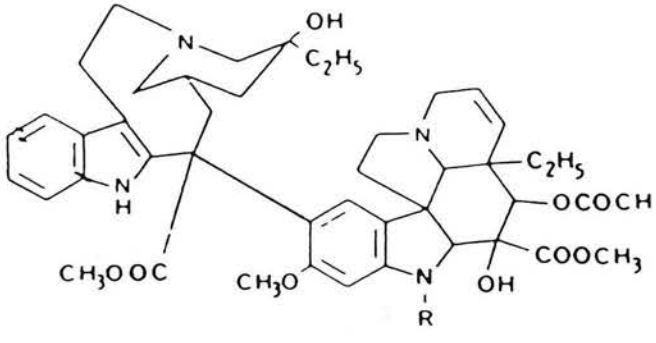
Figure 1.2 Examples of anticancer drugs in the group of compounds classed as vinca alkaloids and antimetabolites



5-Fluorouracil



Cytosine arabinoside



R = CHO **Vincristine**

R = CH_3 **Vinblastine**

DNA formation and repair, and is also directly incorporated into the DNA due to its structural similarity to deoxycytidine. This latter event has the effect of destabilising the DNA chain, making it more susceptible to degradation (Farmer 1985).

(c) The vinca alkaloids.

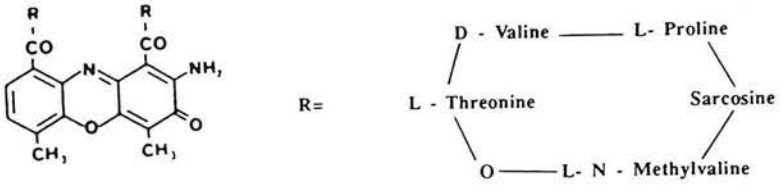
The vinca alkaloids are derived from the periwinkle plant, *Vinca rosea* . The three major drugs in this group are vincristine, vinblastine (Fig 1.2) and vindesine. All have a similar mechanism of action in that they bind to tubulin, the intracellular protein that polymerises to form microtubules. This causes mitosis to halt at the metaphase stage. Although this is probably the major cytotoxic effect,these drugs have also been shown to have a number of other properties including inhibition of thymidine incorporation into DNA and uracil incorporation into RNA. The drugs in this group are those found in the spectrum of compounds associated with the multidrug resistant phenotype (Johnson et al, 1963).

(d) The Antimitotic Antibiotics

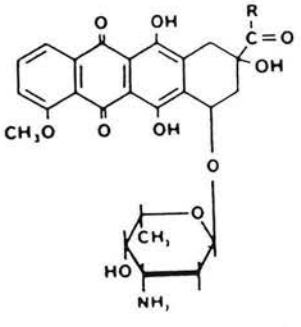
These drugs include the anthracycline antibiotics, daunorubicin, doxorubicin (Fig 1.3) and epirubicin. These compounds are pigmented antibiotics produced by different strains of *streptomyces*. At least four potentially cytotoxic effects of these agents have been identified.

Firstly, the molecular structures of these drugs are such that they can insert themselves between opposing DNA strands; this intercalation disturbs the various DNA functions. Secondly, it has also been shown that these drugs can bind to various membrane components leading to alterations in membrane fluidity and changes in the permeability to various ions. Thirdly, it is known that these agents produce free radicals due to redox cycling of the quinone moiety of their structure. These free radicals are highly reactive and modify DNA as well as other macromolecules. Fourthly, certain metabolites of the anthracycline antibiotics can also act as alkylating agents (Farmer , 1985). The relative importance of these different mechanisms to

Figure 1.3 Examples of anticancer drugs in the group of compounds classed as antimitotic antibiotics.

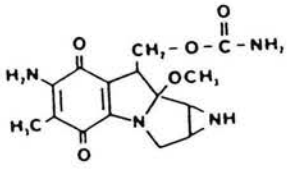


Actinomycin D



R = CH_2OH **Adriamycin**

R = CH_3 **Daunomycin**



Mitomycin C

the antitumour activity of the drugs is still unclear.

Other non-anthracycline antibiotics are exemplified by drugs such as actinomycin D and mitomycin C (Fig 1.3). The former of these compounds is produced by the soil microorganism *actinomyces*. This drug is thought to intercalate with DNA and bind to guanine resulting in cessation of DNA, RNA and ultimately protein biosynthesis.

The mechanism of action of mitomycin C is unclear. It is possible that it acts as an alkylating agent and forms cross-links with DNA. Alternatively, the drug generates free radicals. The net results are alkylation, DNA strand breakage and inhibition of DNA and RNA synthesis.

(e) Miscellaneous Compounds

The anticancer agents that are placed in this catergory include non-classical alkylating agents such as cisplatin and carboplatin. These two compounds carry a pair of chloride atoms which react in particular with the nitrogen atom at the N-7 position of guanine, in the DNA chain. The two chloride atoms are able to react with different guanines in the helical DNA molecule thereby forming both inter- and intra-strand linkages.

Also included in this group of miscellaneous compounds are the anthracenedione drugs such as mitoxantrone, which cannot be described as alkylating agents, antimetabolites, vinca alkaloids or antimitotic antibiotics. They are derived from various chemical dyes and are chemically similar to doxorubicin. The mechanism of action of these compounds remains to be elucidated but mitoxantrone is known to intercalate with DNA causing strand breakage (Koeller & Eble, 1988).

As demonstrated above, the sites and mechanisms of action of commonly used anticancer drugs are diverse and numerous. It would therefore perhaps be naive to think a single mechanism could account for resistance to all these compounds. Those mechanisms thought to be the most likely routes by which tumour cells acquire resistance or remain intrinsically resistant are discussed below.

1.1.2 Mechanisms of Resistance to the Antimetabolites

The antimetabolites are not among those commonly associated with the multidrug resistant phenotype. As discussed earlier, the three main drugs in this group which are used clinically are methotrexate, which is predominantly used to treat choricarcinoma, 5-fluorouracil, used mainly in the management of solid tumours, particular adenocarcinomas of the breast and gastrointestinal tract and cytosine arabinoside, which is used to treat leukaemia. The major problem with these drugs is the onset of resistance to the agent used. Cross-resistance to other anticancer drugs is not often seen.

Resistance to methotrexate can arise by a variety of mechanisms. The enzyme dihydrofolate reductase, which is inhibited by methotrexate, can be overexpressed in tumour cells due to an amplification of the gene coding for this protein (Alt et al, 1978). Alternatively, resistance to methotrexate can occur due to mutations in the gene coding for dihydrofolate reductase (Simonsen & Levinson, 1983). It has also been suggested that mutations in the folate transport carrier, which is responsible for the uptake of methotrexate (Sirotnak, 1985) will also lead to resistance. Several of these mechanisms may be operational in a methotrexate resistant tumour population at any one time.

Resistance to 5-fluorouracil may occur via a similar mechanism to that seen for methotrexate, in that mutations in the carrier system responsible for uptake of the drug (Wohlheuter et al, 1980) will result in resistance. Since 5-fluorouracil must first be activated in order to cause toxicity, loss of the enzyme activity responsible for the conversion of the drug to its active nucleotide, will also lead to resistance to the drug (Ardalan et al, 1980). Alterations in the structure of the target enzyme thymidilate synthetase may also confer resistance to 5-fluorouracil.

1.1.3 Involvement of Topoisomerase II in Drug Resistance.

DNA topoisomerases I and II are known to alter the topology of DNA during replication. DNA topoisomerase II is thought to act by knotting, unknotting, catenation

and decatenation of DNA and relaxation of supercoiled DNA (Gellert 1981; Wang 1985). These topological rearrangements require one double strand of DNA to be passed through another double strand of DNA, which necessitates at some stage in the process, DNA strand breakage. The orientation of the DNA after this break has occurred is maintained by non-covalent bonds between the topoisomerase II enzyme and the DNA.

The enzyme bridges strand breakages that have occurred in complementary strands of DNA. This intermediate in the topological rearrangement of the DNA is called the cleavable complex (Liu 1985; Ross 1985). Certain anticancer drugs are known to interact with topoisomerase II (e.g.vincristine and vinblastine) and freeze the topoisomerase II in covalent association with the DNA (i.e. augment the amount of cleavable complex formed).The exact mechanism of toxicity is unclear, but it is thought to be due to some property of the complex rather than loss of enzyme activity.

The greater the level of expression of topoisomerase II, the more sensitive the cells are to drug-induced cytotoxicity. It is proposed that tumour cells may become resistant by failing to respond to the proposed toxic effects of the cleavable complex upon treatment with topoisomerase II-active drugs. The exact mechanism by which this occurs is unknown, but there are at least three possibilities :

1. Insufficient enzyme so that cleavable complex formation is not possible.
2. Presence of a mutant enzyme which is not stimulated by the drugs to form a fixed complex with the DNA.
3. Regulation of cleavable complex formation.

Whatever the mechanism, it is thought that resistance to this type of drug occurs due to reduced cleavable complex formation. In several cell lines resistant to topoisomerase II active drugs, there has been shown to be lower levels of cleavable complex and lower levels of topoisomerase II (Long et al, 1986; Bakic et al, 1986; Edwards et al, 1987).

1.1.4 P-glycoprotein

The drugs usually associated with the multidrug resistant (MDR) phenotype are vinblastine, vincristine, etoposide (VP16), teniposide (VM-26), doxorubicin (adriamycin), daunorubicin, plicamycin (mithramycin) and actinomycin D. Although these drugs do not have common targets within tumour cells, they are all hydrophobic drugs derived from natural products (Safa et al, 1987). The alkylating agents and antimetabolites are not normally in the spectrum of drugs associated with MDR, although resistance to these drug classes also presents a considerable problem in cancer treatment.

The development of *in vitro* cell lines established from *in vivo* tumours has aided the study of drug resistance. Many cell lines have now been described that exhibit the MDR phenotype. These have all been derived by sequential selection in an increasing concentration of a chosen anticancer drug and by definition display resistance to a range of other antineoplastic agents. Biedler & Riehm (1970) were the first workers to report that the MDR phenomenon encountered clinically, could be mimicked *in vitro* using cell culture models.

In the 1970's Ling and co-workers derived a Chinese hamster ovary line resistant to colchicine (Juliano & Ling, 1976) and showed this cell line exhibited cross-resistance to a range of anticancer drugs. The resistant cell line was found to exhibit a decreased accumulation of drug. These workers showed that the decreased drug accumulation exhibited by the resistant cells was associated with the presence of a membrane glycoprotein of molecular weight 170,000 daltons, (Juliano & Ling, 1976; Bech-Hanson et al, 1976). This membrane glycoprotein has been termed P-glycoprotein and since its discovery much effort has been directed towards defining its role and contribution to the drug-resistant phenotype. Much of the available evidence shows a strong correlation between P-glycoprotein expression and the MDR phenotype.

Several cDNA clones encoding P-glycoproteins have been isolated and sequenced from a variety of mammalian species (Chen et al, 1986; Gros et al, 1986.) Analysis of the DNA sequences of these clones has shown that the P-glycoprotein

genes can be divided into two classes on the basis of sequence homologies. These are MDR 1 and MDR 2. Of the two classes only the former can confer drug resistance. This has been demonstrated using gene transfection studies in which MDR 1 genes have been expressed in drug sensitive lines and resistance to the spectrum of drugs commonly associated with the MDR phenotype has been demonstrated (Gros et al, 1986; Ueda et al, 1987; Croop et al, 1987; Pastan et al, 1988).

How P-glycoprotein confers drug resistance to the wide spectrum of drugs commonly associated with drug resistance is not certain. The functional aspects of the protein that produce resistance will surely provide valuable insight to aid novel drug design. Much of the evidence now available suggests that P-glycoprotein functions as an energy dependent drug-efflux pump.This evidence is described below.

Firstly, from gene sequence analysis it has been shown that P-glycoprotein shares homology with several bacterial transport proteins (Ueda et al, 1986). Haemolysin B, with which P-glycoprotein has been shown to share closest homology, is responsible for transporting alpha-haemolysin out of certain bacteria. However, homology with Mal K, which is part of the maltose transport system in *Escherichia coli* and His P which is part of the histidine permease system has also been noted (Gerlach et al, 1986).It has been suggested that this glycoprotein could possibly have arisen from tandem duplication of an ancestral gene, as it contains two regions homologous to the nucleotide binding domains on the energy transducing subunits of these bacterial transport proteins (Gottesman, 1988). Secondly, when MDR cell lines are grown in the presence of metabolic inhibitors such as 2-deoxyglucose and azide, which are known to deplete intracellular ATP, drug resistant cells can accumulate cytotoxic agents to levels approaching the parental sensitive line (Croop et al, 1988). When the inhibitors of ATP production are removed there is a rapid efflux of the cytotoxic agent out of the cell.

Hammond and co-workers (Hammond et al, 1989) have more recently provided evidence showing that the MDR 1 gene product is responsible for the mechanisms controlling cellular drug levels in an ATP-dependent manner. In chinese hamster ovary (CHO) cells stably transfected with and overexpressing mouse MDR 1, a decrease in

[^{3}H] vinblastine release was seen which was arrested when cellular ATP levels were reduced.

Other workers using photoactivatable derivative of vinblastine has shown that P-glycoprotein is able to bind this drug (Cornwell et al, 1986). Although much of the data available suggest a strong correlation between P-glycoprotein and the MDR phenotype *in vitro*, there is information available which suggests that this is not the only mechanism involved in drug resistance.

The binding constants of a variety of drugs with P-glycoprotein is estimated to be in the micromolar range (Cornwell et al, 1986), which is higher than the drug concentration range clinically achievable. This sort of information raises questions about the *in vivo* significance of P-glycoprotein as does the clinical data that has become available in recent years.

Each *in vitro* model, although apparently showing overexpression of P-glycoprotein, displays a different pattern of cross-resistance (Moscow & Cowan, 1988). Although several P-glycoprotein genes have been reported (Van der Bliek et al, 1987; Gros et al, 1986) and there may be variation in expression of these genes, these two things taken together do not account for the diversity seen in cross-resistance patterns. Still more data that raises questions as to the importance of P-glycoproteins, is the fact that the degree of resistance in drug-resistant cell lines is not directly correlated with the intracellular drug accumulation (Louie et al, 1986; Sirotnak et al, 1986).

Mukhopadhyay & Kuo (1989), have looked at MDR 1 copy number and mRNA levels during the derivation of a vincristine resistant CHO cell line. They found high levels of MDR 1 in cells showing low levels of resistance when compared to the wild-type cell line. However, as resistance to vincristine increased, the P-glycoprotein levels did not, suggesting other mechanisms of resistance may be coming into operation in this model.

Other *in vitro* models also support the presence of alternative resistance mechanisms. McGrath & Center, (1987) derived an adriamycin resistant human leukaemia cell line (HL6O) that exhibits the MDR phenotype but shows no detectable levels of P-glycoprotein. Proteins conferring resistance in this cell line may be different

from those previously described (Beck et al, 1987; Fergusson et al, 1988). Other cell lines displaying resistance to various anticancer drugs without expression of P-glycoprotein have also been reported (Danks et al, 1987).

The *in vivo* patterns of resistance to anticancer drugs frequently differ from those observed in drug resistant tissue culture cell line models. Sometimes in addition to resistance towards the typical drugs that are associated with MDR, resistance towards alkylating agents and antimetabolites is also noted. Recent clinical evidence on MDR 1 expression in human tumour samples has yielded information suggesting great diversity of P-glycoprotein expression in different tumours (Bell et al, 1985; Gerlach et al, 1987; Ma et al, 1987). The picture generated by these workers about the role of P-glycoprotein in the patient, is far from clear-cut.

Essentially, P-glycoprotein is expressed in some tumours refractory to chemotherapy but not in others which are equally refractory. There seems to be little correlation between P-glycoprotein expression in tumours analysed before or after treatment, this being so particularly in the case of certain lung tumours (Lai et al, 1989). Recently, in a large study, P-glycoprotein expression in over 400 tumour samples of diverse origin were analysed (Goldstein et al, 1989). This study indicated that there was a consistent association of MDR 1 expression in several intrinsically resistant cancers and also increased expression of the MDR 1 gene in certain cancers with acquired drug resistance. Absence of detectable P-glycoprotein in other intrinsically resistant tumours suggested the occurrence of other resistance mechanisms.

1.2.0 THE GLUTATHIONE S-TRANSFERASES

Although it is clear from the preceding section that resistance to anticancer drugs may be attributable to several known mechanisms, it is clear that other important mechanisms still remain to be fully elucidated. It has been proposed that the glutathione S-transferases may have the ability to detoxify alkylating anticancer drugs since they are known to catalyse the conjugation of xenobiotic electrophiles to glutathione. The GSTs may also bind non-covalently to drugs and carcinogens thus also inactivating them via this route (Hayes & Wolf, 1988).

The GSTs were initially discovered simultaneously by Combes & Stakelum (1961) and Booth et al (1961) who were studying, respectively, the metabolism of bromosulphophthalein and 1,2-dichloro-4-nitrobenzene in rat liver. These enzymes are multifunctional and because of their diverse activities they have enjoyed various names. Owing to their ability to bind non-substrate ligands such as bilirubin (Levi et al, 1969) GSTs have also been called 'ligandins' (Litwack et al, 1971).

GSTs have subsequently been shown to be ubiquitous in nature, being found in many organisms ranging from bacteria to man. In the three mammalian species studied in the greatest detail, man, rat and mouse, the complexity of this enzyme system has become evident. The different enzymes in these species have been shown to be encoded by multiple genes which are subject to complex regulation.

All GSTs so far discovered can catalyse the formation of a thioether bondbetween reduced glutathione and a large number of reactive electrophiles. In addition to this function, certain enzymes also have peroxidase activity. One other cellular function that certain GSTs perform, is binding to non-substrate ligands which may aid excretion of certain compounds from the body. The large spectrum of electrophilic compounds, many of them potentially harmful, with which the GSTs can interact is achieved through the range of isoenzymes present in cells. Each particular isoenzyme has its own particular range of substrate specificities.

The literature concerning the glutathione S-transferases (GSTs) is extensive and somewhat confusing. This confusion stems from the fact that the relationship between the multiple forms isolated from different species has not always been clearly defined. With the development of molecular biology and protein sequencing techniques, it has now become clear that the cytosolic GSTs from mouse, rat and man can be grouped into three classes or families, which have been named alpha, mu and pi (Mannervik, 1985; Mannervik et al, 1985). In addition, a microsomal protein has also been identified (Morgenstern et al, 1980).

This grouping is based on several parameters. Firstly, sequence data at both the DNA and protein level has revealed strong homology between isoenzymes within a class isolated from the three different species (Mannervik & Danielson, 1988). Secondly, studies using GST substrates have shown that these enzymes can be divided

into the three groups on the basis of their substrate specificities (Jakoby, 1978; Chasseaud, 1979). This classification system also applies when various inhibitors of the enzymes are used (Mannervik & Danielson, 1988; Boyer, 1986 ; Boyer, 1989). Thirdly, using immunochemical techniques such as Western blotting (Hayes & Mantle, 1986; Hayes et al, 1987 a ;b), antibodies raised against a purified protein isolated from one of the three groups can be shown to cross-react only with other proteins within the same group.

The most extensively studied GST isoenzymes are those isolated from the rat and although this thesis will concentrate on human GSTs, it will be necessary to make frequent reference to the rat counterparts. All cytosolic GSTs so far isolated consist of homo or hetero dimeric combinations of various GST subunits.

1.2.1 GST Isolation Procedures

The development of affinity matrices has simplified the purification of the GSTs in rat, mouse and man. The two most commonly used affinity gels are S-hexylglutathione-Sepharose affinity matrices (Mannervik & Guthenberg, 1981) and glutathione-Sepharose matrices (Simons & Vander Jagt, 1977; Vander Jagt et al, 1985). The pools of GST activity isolated by the above methods can be resolved into the individual isoenzymes using other chromatographic techniques such as chromatofocusing, ion exchange and hydroxyapatite columns. Alternatively, subunits can be resolved by reverse phase high pressure liquid chromatography (Ostlund Farrants et al, 1987).

The S-hexylglutathione-Sepharose affinity matrix has been the most commonly used technique. In rat, mouse and man there is an ordered elution of the subunits from chromatography columns. This may reflect the conservation of activities of the different subunits between the species and is likely to be mediated by the GSH-binding site in that different forms from the same class will show different affinities for the glutathione affinity matrix (Hayes, 1988).

Using the techniques outlined above, multiple GST forms have been isolated (Reddy et al, 1983; Guthenberg et al, 1985; Stockman et al, 1985; McCusker and

Mantle, 1987; Warholm et al, 1986; Hayes & Mantle, 1986; Hayes, 1986; Hayes et al, 1987 a;b.) and the methodologies are applicable to the GSTs from many species. However, it is becoming evident that in rat there are a significant number of GSTs which remain to be purified. This is also likely to be the case for other species (Hussey et al, 1990). Care should be taken in interpreting the results from different laboratories. Claims of new GST forms should stand up to rigorous scrutiny and it is highly desirable that molecular evidence for the existence of novel forms of GST should be presented.

1.2.2 Classification

Tables 1.1 to 1.3 show how the rat, mouse and human cytosolic GSTs have been assigned to the alpha, mu and pi classes. As already stated, this has been based on substrate specificities, immunological analysis and sequence data. The latter of these techniques have provided the most informative data about the molecular relationship between the GST forms.

The primary structures from both protein sequence and DNA sequence data for different GSTs has been compared for the three species (Mannervik & Danielson, 1988). There was shown to be close homology between enzymes within a class when the amino acid sequences were aligned. These data have lead to speculation that the GSTs evolved into separate classes before the divergence of the mammalian species.

1.2.3 GST Nomenclature

(a) Rat cytosolic GST nomenclature

Jakoby and co-workers (Habig et al, 1974a; Habig et al, 1976; Jakoby et al, 1976) originally isolated six forms from rat liver, all with a neutral or basic isoelectric point. They were named alphabetically E, D, C, B, A and AA, on the basis of their elution order from a carboxy-methylcellulose ion-exchange matrix. Each enzyme was found to possess overlapping substrate specificity.

Subsequently, Mannervik and his co-workers isolated six major GST forms from rat liver (Mannervik & Jensson, 1982) and showed these dimeric proteins to comprise homo- and hetero-dimeric combinations of four separate subunits, each displaying distinct substrate specificities.

Table 1.1 Nomenclature for human GST enzymes

GST ISOENZYME	FAMILY	SUBUNIT MOLECULAR WEIGHT (By SDS/PAGE)	ISOELECTRIC POINT	REFERENCES
(1) B_1B_1 (8) (ε)	Alpha	25,900	8.9	(1) Stockman et al, (1985), (8) Kamisaka et al, (1975)
(1) B_1B_2 (8) (γ)	Alpha	25,900	8.75	
(1) B_2B_2 (8) (γ)	Alpha	25,900	8.4	
(2) Skin "9.9"	Alpha	27,500	9.9	(2) Del Boccio et al, (1987)
(3) μ	Mu	26,700	6.1	(3) Warholm et al, (1983)
(4) ψ	Mu	26,700	5.5	(4) Hayes (1989a)
(5) φ	Mu	26,700	4.6	(5) Stockman & Hayes, (1987)
(6) π	Pi	24,800	4.8	(6) Guthenberg et al, (1979)
(7) Microsomal	Not given	17,300	Not determined	(7) McLellan et al, (1989)

Table 1.2 Nomenclature for rat GST enzymes

GST ISOENZYME		FAMILY	SUBUNIT MOLECULAR WEIGHT (By SDS/PAGE)[1-7]	REFERENCES
"Y" designation[1-6]	Numerical Designation[5]			
(1) Ya	1	Alpha	25,500	(1) Hayes (1988)
(1) Yc	2	Alpha	27,500	
(1) Yk	8	Alpha	25,500	
(1) Yl	10	Alpha	25,700	
(1) Yb_1	3	Mu	27,500	
(1) Yb_2	4	Mu	26,500	
(3), (4) Yb_3	6	Mu	26,000	(3) Abramovitz & Litowsky (1987) (4) Guthenberg et al (1985)
(1) Yn_2	9	Mu	26,000	
(5) Yo	11	Mu	26,500	(5) Kispert et al (1989)
(6), (7) Yf or Yp	7	Pi	24,800	(6) Kitahara et al (1984) (7) Hayes & Mantle (1986)

Table 1.3 Nomenclature for mouse GST enzymes

GST ISOENZYME (Refs 1-3)			FAMILY	SUBUNIT MOLECULAR WEIGHT (By SDS/PAGE) (Refs. 1- 3)	ISOELECTRIC POINT (Refs.1 - 3)
"M" Designation	Numerical Designation	"Y" Designation			
MI	4-4		Alpha	26,000	9.2
		YaYa	Alpha	25,000	9.7
MIII	1-1 or 2-2	YbYb	Mu	27,000	7.8-8.2
MII	3-3	YfYf	Pi	24,500	8.6

References: **(1)** Warholm et al, (1986); **(2)** McLellan & Hayes (1987); **(3)** Hayes et al, (1987b).

GSTs from rat liver have been isolated independently by other groups (Sheehan & Mantle, 1984; Hayes, 1986; Hayes et al, 1987 a;b; Hayes & Mantle, 1986) and these have been given nomencltures based on their mobility on SDS-PAGE (Bass et al,1977) called Ya, Yb, Yc etc. A more recent system of nomenclature has been based on Arabic numerals. Table 1.2 shows the different subunits isolated to date and compares the Ya, Yb etc., nomenclature with the Arabic numeral system.

(i) Rat Class Alpha

To date five class alpha GSTs have been isolated from various rat tissue. These are: YaYa (1-1), YcYc (2-2), YaYc (1-2) (Hayes et al , 1981; Hayes 1988), YkYk (8-8) (Hayes, 1986) and Y1Yc (10-2) (Scott & Kirsch, 1987; Hayes 1988). The tissue distribution of these different forms may reflect the function of the specific tissue and the range of electrophilic compounds with which a particular tissue may come into contact.

(ii) Rat Class Mu

This class seems to contain the most isoenzyme forms and to date the following isoenzymes have been discovered in different rat tissues: Yb_1Yb_1 (3-3), Yb_2Yb_2 (4-4), Yb_1Yb_2 (3-4), Yb_1Yn_1 (3-6), Yb_2Yn_1 (4-6), Yn_1Yn_1 (6-6) Yn_1Yn_2 (6-9), Yn_2Yn_2 (9-9) and YoYo (11-11) (Hayes, 1988; Kispert et al, 1989).

(iii). Rat Class Pi

To date only one subunit in this group has been isolated and this was from rat placenta. This is subunit YfYf (7-7) and is sometimes also referred to as YpYp (Kitahara et al, 1984).

1.2.3 (b) Mouse Cytosolic GST Nomenclature

GSTs isolated from the mouse have also been named using Arabic numerals and classified using the same criteria for substrate specificity, amino acid sequence and immunological properties as GST from rat and man.

The subunits isolated to date are not as extensive as for rat or man and are shown in Table 1.3. Although the subunit designations Ya, Yb and Yf have been used to describe the equivalent polypeptides in rat and mouse (McLellan & Hayes 1987) the numerical classification system used for mouse is independent of that used for rat. This may lead to confusion since mouse enzyme 3-3 is not equivalent to rat 3-3 (Mannervik & Danielson, 1988).

1.2.3 (c) Human Cytosolic GST Nomenclature

The human GSTs on which the rest of this thesis will concentrate, have been shown to consist of a variety of forms that can be isolated from a range of human tissues. Again the literature concerning the human GSTs is diverse and the nomenclature somewhat confusing owing to the independent isolation of identical forms by different research groups and a subsequent independent naming system by individual groups. The system used today is shown in Table 1.1.

(i). Human Class Alpha

Kamisaka et al, (1975), isolated five GST forms of basic pH from human liver cytosol, originally termed α, β, γ, δ, ε in order of increasing isoelectric points. It was subsequently difficult to correlate these enzyme forms with other basic forms isolated by other workers. As a result of the confusion as to their true identity, the forms isolated by Kamisaka and his colleagues have sometimes been referred to as the (α–ε) (Mannervik & Danielson, 1988).

Later work has shown the human cytosolic GSTs from the class alpha family to comprise of two homodimers and a heterodimer formed by two different subunits B_1 and B_2 (Stockman et al, 1985; 1987; Hayes et al, 1990). It has been proposed that B_1B_1, B_1B_2 and B_2B_2 correspond to the GST ε, δ and γ isolated by Kamisaka and co-workers (Stockman et al, 1985; 1987).

Recently a GST isolated from human skin has also been assigned to this class (Del Boccio et al, 1987) and termed 9.9. It has not been reported in any other tissue to date.

(ii). Human Class Mu

The human enzymes in this group have pI values of about 4.5-6.5 and were initially referred to as the neutral GST group. A more rational classification based on the aforementioned criteria, has shown there to be at least three subunits in this class these being subunits μ, φ, and ψ (Stockman & Hayes 1987; Hayes 1989). Recently, data has been presented showing that at least three forms of class mu enzymes are present in human skeletal muscle (Hussey, et al, 1990).

It is not known whether enzymes in this group all exist as homodimers or whether they can also form heterodimers. Heterodimers consisting of μ and ψ subunits have been demonstrated *in vitro* (Hayes 1989). There is now one report in the literature of a μ-ψ heterodimer being present in human liver (Van Omenn et al, 1989).

(iii). Human Class Pi

To date, it appears that there is only one enzyme form that can be assigned to the class pi group (Guthenberg & Mannervik, 1979). This was originally isolated from placenta but has been observed in a variety of other tissues. Although another class pi GST form has never been isolated it is noteworthy that fatty acid ethyl ester synthetase from human myocardium has been reported to be highly homologous, but not identical to the placental pi GST (Bora et al, 1989).

1.2.4 Polymorphic Expression of Human Cytosolic GSTs

There is marked polymorphic expression of the GST enzymes. The response of an individual's tissues to cytotoxic insult is likely to be determined by the drug detoxification capacity of that tissue. Since many of the reactions catalysed by the GSTs serve to detoxify reactive metabolic intermediates, it may be speculated that the lack of certain GST isoenzymes may predispose an individual to carcinogenesis since certain carcinogens may not be detoxified efficiently.

The multiplicity of GST forms is indicative of a multigene family. To date, at least three loci have been defined that code for members of the three cytosolic classes (Board 1981; Strange et al, 1985). As yet it is not clear how many genes there are in each family but it is thought that the multiple isoenzymes arise to some extent from allelic variance.

(a) GST 1 Locus

GST 1 locus encodes members of the human class mu enzymes . This locus has so far been shown to consist of three common alleles.

GST 1 type 1 and GST 1 type 2 both encode similar protein products, the former coding for the ψ subunit, the latter coding for the μ subunit. GST 1 type 0 encodes no protein product since it appears to have resulted from a gene deletion. One of the proteins encoded by the GST 1 locus has been shown to be polymorphic in man. 40% of the human population fail to synthesise the μ homodimers (Seidegard et al, 1986). Seidegard et al (1990) have shown this to be due to a deletion in the mu gene. Evidence has also been presented that individuals nulled for GST μ are more susceptible to lung cancer if they smoke (Seidegard et al, 1986).

It seems likely, in the light of recent data (Hussey et al, 1990), that multiple forms of the human mu-class GST family exist. The fact that human mu-class genes have mapped to both chromosomes 1 (De Jong et al, 1988) and 3 (Islam et al, 1989) is

in itself interesting and suggests the presence of more than one locus which could account for the multiplicity of class-mu proteins.

(b) GST 2 Locus

This locus codes for the class alpha proteins and has been shown so far to contain at least two closely linked genes referred to as GST 2 type 1 and GST 2 type 2. The former has been shown to code for GST B_1B_1 and the latter for B_2B_2. Recent work has compared fifty percent of the protein sequence of the human B_1 and B_2 subunits (Hayes et al, 1990) with cDNAs for class alpha proteins, isolated from human liver. This work has shown that these two subunits are 96% homologous but show differences at amino acid residues 88, 110, 111, 112, 116, 124 and 127, indicating that they are indeed different gene products and that their expression is therefore unlikely to be coordinately regulated. The GST 2 locus has been shown to map to chromosome 6 (Board & Webb, 1987).

The expression of subunits from these genes has been shown to be variable in man (Hussey et al, 1986; Singh et al, 1987). The basis of the inter-individual differences in the expression of B_1 and B_2 has yet to be discovered.

(c) GST 3 Locus

So far the available data has shown that this locus encodes a single gene (Cowell et al, 1988; Morrow et al, 1989) which is expressed in most tissues so far analysed. This locus is found on chromosome 11 (Laisney et al, 1983). The polymorphic expression of the gene product is open to question.

(d) Relationship of Gene Loci to Protein Products

The relationship of the gene loci to the actual protein product is not obvious. Although GST loci 1,2 and 3 are now known to contain genes coding proteins in the mu, alpha and pi classes, there are still other gene loci whose genes and subsequent protein products are still difficult to classify. For example the GST-4 locus encodes a near neutral enzyme and has close homology with GST 1 (Board et al, 1988).The

GST-5 locus encodes a protein product found in brain which is also closely homologous to GST 1.

The progress at the genetic level has been slower than that at the protein level. The genetic information available at present makes it impossible to say how many genes there are per family and what their actual protein products are. It is clear that more work is needed in this area to complement the work at the protein level with molecular data.

1.2.5 Tissue Distribution of Human Cytosolic GSTs

In man these enzymes have been most extensively studied in liver (Stockman et al, 1985; 1987; Boyer & Kenney 1985; Singh et al, 1985; Hussey et al, 1986; Soma et al, 1986; Tu & Quian 1987; Singh et al, 1987; Board & Pierce 1987). Interest in the tissue distribution of human cytosolic GSTs is fuelled by the fact that this may be a determinant of resistance or sensitivity of a given tissue to certain drugs and carcinogens.

Because the GSTs have been shown to have a wide range of substrate specificities (see Section 1.2.7), it would be expected that organs involved in detoxification processes may possess more GST activity and perhaps a wider range of GST isoenzymes than organs which are not involved with drug metabolism.

It has proved difficult to generalise about the GST content of various human tissues since expression of some of the isoenzymes shows a marked polymorphism. However, whilst comments about the expression of specific subunits is unwise, a few generalisations about the distribution of the three classes of GST in different human organs can be made and there is an increasing body of literature concerning this subject.

(a) Human Class pi GST

This enzyme has a wide tissue distribution and although levels of expression may vary in the different tissues. The pi class enzyme has been detected in lung, bladder, breast (Ketterer 1988), kidney (Sherman et al, 1983; Tateoka et al, 1987; Singh et al, 1987; Pemble et al, 1987), erythrocytes, platelets, thyroid, heart, spleen

(Koskelo 1983; Di Ilio et al, 1986; Tateoka et al, 1987), and at low levels in liver (Mannervik, 1985; Hussey et al, 1986).

Peters et al (1989) have looked at human intestinal GST π and found little variation in this enzyme level from proximal to distal sections of the small intestine. These workers reported GST π to be present in large amounts in the normal mucosa of small and large intestine. Kodate et al (1986) have looked at mRNA levels of π in normal human colon and found there to be high expression. Moscow and co-workers (Moscow et al, 1989) have carried out a comparative study on π mRNA levels in various normal human tissues.They found the level of expression is heterogeneous, the highest levels being seen in lung, oesophagus and placenta and the lowest levels being detectable in liver. The low levels of expression found in liver by these workers is in keeping with the results of other research groups.

(b) Human GST Class alpha

Of all tissues examined, this enzyme class is expressed predominantly in human liver (Stockman et al, 1985). The subunits detected so far in human liver have been B_1 and B_2 either as GST B_1B_1, B_1B_2 or B_2B_2. These isoenzymes have also been detected in human adrenal, ovary and testis tissue (Sherman et al, 1983; Corrigal & Kirsch, 1988). They have also been found in kidney (Di Ilio et al, 1987). Another GST form isolated from human skin (9.9) is also thought to belong to the class alpha enzyme group (Del Boccio et al, 1987). However, with the possible exception of cardiac muscle this isoenzyme has not been recorded in any other human tissue (Di Ilio et al, 1986).

(c) Human GST Class mu

The tissue studied in the greatest detail with respect to these enzymes, is again the liver. The subunits found to date in this tissue are μ (Warholm et al, 1983), ψ (Hayes et al, 1987) and ϕ (Stockman & Hayes, 1987) and as already stated, there is

polymorphic expression of both ψ and μ (Van Ommen, 1989; Seidegard et al, 1986). Of other tissues examined, the μ homodimer has been found in human mononuclear leukocytes (Seidegard et al, 1987). Of the three human cytosolic classes, the class mu is the least well characterised in terms of tissue distribution. It remains to be seen whether there is a marked tissue specific expression of enzymes in this class. Recent evidence (Hussey et al, 1990) has revealed the presence of several class mu enzymes in human muscle tissue termed N_1, N_2 and N_3. The primary structure of N_1 has shown it to be homologous to the μ subunit. It remains to be seen whether the N_2 and N_3 subunits have homologues in other human tissues.

It is apparent that more work needs to be done in this area in terms of identification of the particular subunits expressed in human tissues. As well as this, the role of these isoenzymes in each particular tissue needs to be established. It is important to know the catalytic functions of each subunit in order to assign specific physiological roles to the different isoenzymes within a given tissue.

1.2.6 GSTs as Intracellular Transport Proteins

The binding of GSTs to non-substrate ligands, such as bilirubin, led to their earlier name of ligandin (Levi et al, 1969; Litwack et al, 1971). They have subsequently been shown to bind a range of compounds including steroids and bile acids. A number of other non-physiological inhibitors are also able to bind to GST and some of them have been used, along with other criteria, to assign the GSTs into the alpha, mu and pi classes. This has been possible because isoenzymes within a group have distinct affinities for these compounds How non-substrate ligands exert their inhibitory effect on enzyme function is unknown. It has been suggested that the substrates may bind at the active site or at an alternative specific binding site (Boyer, 1989). The physiological role of this phenomenon has been an area of active research for many years (Wolkoff et al, 1979; Tipping & Ketterer 1981). It has been suggested that the GSTs may play a role in the transport of non-polar or alipathic molecules by binding to them and rendering them more soluble (Husby et al, 1981; Senjo et al,

1985). More recently, a non-histone binding protein isolated from rat was found to be a class mu enzyme (Bennett et al, 1986).

There have also been suggestions that GSTs may function as steroid binding proteins. Experiments with dexamethasone 21-methane-sulphonate have shown that class mu GST isolated from rat nuclei is able to bind to this compound (Homma & Listowsky, 1985). Due to the binding capabilities of the GSTs it is feasible that they may bind harmful chemical species and aid their excretion before the reactive chemicals can cause cellular damage.

1.2.7 Reactions Catalysed by the GSTs

The GSTs have a broad range of substrate specificities owing in part to the existence of multiple isoenzymes. More is known about the exogenous substrates since it is these that have been used diagnostically in classifying the GSTs. However, many of the diagnostic substrates are not encountered *in vivo* and so have little biological significance (see Table 1.4).

(a) Exogenous Substrates

Many exogenous substrates for GST such as herbicides, pesticides and pollutants from combustion of organic material, are widely encountered in the environment. Many of these compounds have been shown to have carcinogenic properties and so it is desirable that if they are ingested they be detoxified and removed from the body. Conjugation of toxic xenobiotics with glutathione (catalysed by the GSTs) represents a major detoxification pathway, the final product of detoxification being mercapturic acid, which is excreted (Gibson & Skett, 1986) .

Environmental pollutants such as 1-nitropyrene-4,5-oxide and 1-nitropyrene-9, 10-oxide are known substrates for GSTs. The actual GST isoenzymes responsible for catalysing these reactions are unknown at present (Ketterer, 1988).

Table 1.4 Substrate Specificities (μM/min/mg at 37°C)

SUBSTRATE	ALPHA			MU			PI
	B_1B_1	B_2B_2	Skin "9.9"	μ	φ	ψ	π
1-Chloro-2-4-dinitrobenzene	82	80	-	261	-	254	105
1,2-Dichloro-4-nitrobenzene	0.25	0.80	-	0	-	0	0.11
Bromosulfophthalein	-	-	0	<0.002	-	-	<0.002
Ethacrynic acid	0.11	0.14	0.31	0.08	-	0.09	0.86
trans-4-Phenyl-3-buten-2-one	0	0	0	0.22	-	0.25	0.01
Leukotriene A_4	-	-	-	0.44	-	-	0.002
trans-Stilbene oxide	0.0006	0.00001	-	5.2	-	-	0.0024
Benzo(a)pyrene 4,5-oxide	-	-	-	0.92	-	-	0.13
Benzo(a)pyrene 7,8-diol-9,10-epoxide	-	-	-	0.57	-	-	0.83
Cumene hydroperoxide	3.1	10.4	4.3	0.22	-	0.20	0.03
Hydrogen peroxide	-	-	-	<0.01	-	-	<0.01
Δ^5-Androstene-3,17-dione	-	-	-	0.12	-	-	-
P-Nitrophenyl acetate	0.66	0.24	-	0.10	-	0.10	-

References: Mannervik and Danielson (1988); Warholm et al (1983); Stockman et al (1987); Del Boccio et al (1987); Robertson & Jernstrom (1986); Hayes (1989a).

A well known carcinogen which is the product of microsomal oxidation of benzopyrene is benzo[a]pyrene-7,8-diol-9,10-oxide (BPDE). This has been shown to be a substrate for all the GST isoenzymes so far tested but particularly for the Yb_2 subunit and the class pi transferase (Nemoto et al, 1975). Alpha class GSTs have been shown to have low activities with this substrate (Robertson & Jernstrom, 1986).

A compound which has been shown to be a powerful hepatocarcinogen in rat is a metabolite of the mould toxin aflatoxin B_1 (AFB_1). Kensler et al, (1986) have shown that induction of a Ya subunit in rat by the antioxidant ethoxyquin, results in increased detoxification of AFB_1-8,9-oxide, the carcinogenic metabolite of AFB_1. Although there is as yet no direct evidence that the conjugation of glutathione to AFB_1 -8, 9-oxide is carried out enzymatically, the fact that induction of a Ya subunit leads to increased biliary excretion of an AFB_1 - GSH conjugate and that there is decreased DNA damage, implicates the Ya subunit in the detoxification process.

A wide range of compounds have been shown to induce GSTs in the rat (Talalay et al, 1988). The inducers include antioxidants, like butylated hydroxyanisole and also ethoxyquin, barbiturates, epoxides, such as trans-stilbene oxide, aromatic hydrocarbons (Igarashi et al, 1987) and N,N-dimethyl-4-aminoazobenzene (Listowsky et al, 1988). In the rat it is predominately Ya (subunit 1) Yb_1 (subunit 3) and Yb_2 (subunit 4) that are induced by these compounds. The induced isoenzymes act to detoxify reactive electrophilic forms of carcinogens.

Recent work by Talalay et al (1988) has highlighted the fact that all the GST inducers are Michael reaction acceptors. It remains to be resolved whether all GST inducers are also themselves substrates for the enzymes. It is possible that the requirement for an inducer to serve as a substrate for GST is an intrinsic aspect of the induction mechanism.

One other important consequence of the detoxification reactions catalysed by the GST, apart from the removal of harmful exogenous carcinogenic products from the body, is that of resistance to anti-cancer drugs. Although at present there is little evidence that many of the commonly used anticancer drugs are potential GST substrates, there is a large body of circumstantial evidence showing that certain GST isoenzymes are elevated in cell lines resistant to a range of anti-cancer agents (see

Section 1.3).

However, there is now evidence that two of the drugs used in chemotherapy at least, are indeed substrates for certain GSTs. Dulik et al (1986) have shown that a melphalan-glutathione conjugate is formed due to enzymatic catalysis by both microsomal and cytosolic GSTs.

More recently Smith et al (1989) have shown that a rat class mu enzyme is effective in the inactivation of BCNU through a denitrosation reaction. In addition to this, over expression of this mu class enzyme in rat brain tumour cells resistant to BCNU was reported.

(b) Endogenous Substrates

One physiological function of the GSTs that has already been discussed is the transport of compounds in the body. As already stated, many of the known GST substrates are of little biological importance. However, many harmful compounds are produced as a result of normal metabolism and are known to be substrates for the different GST classes. For example, certain products of lipid peroxidation such as cholesterol-α-oxide have known mutagenic properties (Meyer and Ketterer, 1982) and have been shown to be particularly good substrates for the alpha class, rat Ya subunit. Breakdown of arachidonate and linoleate yield the lipid peroxides arachnidonate hydroperoxide and lineoleate hydroperoxide. These two compounds have activities with the class alpha GSTs that are comparable to the diagnostic substrate cumene hydroperoxide.

Other major breakdown products of peroxidative degradation of polyunsaturated fatty acids are alkenes such as 4-hydroxynon-2-enal (Alin et al, 1985). In a study by Danielson et al (1987), a series of 4-hydroxyalkenals ranging from 4-hydroxypentenal to 4-hydroxypentadecanal were used as substrates for rat enzymes from the three different classes. The rat alpha class enzyme YkYk (8-8) was most active with the whole range of hydroxyenals.

More recently it has been shown that the products of DNA peroxidation, DNA hydroperoxides, are excellent substrates for the class mu enzymes (Tan et al, 1988). These workers isolated a subunit from rat liver nuclei, termed subunit 5*-5* since it is

unlike any subunit so far isolated. GST 5*-5* showed the highest activity towards DNA hydroperoxides. These workers discussed a possible role for GSTs in repair of damaged DNA.

1.2.8. Regulation of the GSTs

As already mentioned, the tissue distribution of the GSTs has consequences for detoxification of potentially harmful compounds. It is now known that as well as the intrinsic composition of isoenzymes, cells also have the potential to transcriptionally activate genes coding for certain GSTs in response to a range of harmful compounds. This may have consequences for cytotoxic insult by, for example, carcinogens or anticancer agents which are highly toxic molecules.

In recent years there has been much interest in the regulation of the GSTs (Satoh, 1989; Pickett & Lu, 1989). Much of the work has been carried out using rat models and care must be taken in drawing parallels between rat and man. Although GSTs within a class share close sequence homology between species, the regulatory mechanisms may be very different. Since most of the available data relates to the rat system, frequent reference will be made to this. The situation in man will be reviewed where possible.

(a) Structure and Regulation of the Class Alpha GSTs

i. Structure

The structural and regulatory analysis of this class of GST has come predominantly from cDNAs encoding the rat Ya and Yc subunits (Rothkopf et al, 1986; Tu & Quian, 1987). It is becoming clear that the alpha class gene family in rat and probably also in man is very complex. Sequence analysis of the cDNAs for Ya and Yc have revealed them to be 75% homologous in the coding region. However, homology in the 5' and 3' untranslated region is low (Rothkopf et al, 1986). Rothkopf et al (1986) have used cDNAs coding for Ya (pGTB 38) and Yc (pGTB 2) to probe

Southern blots of rat genomic DNA. They showed the presence of at least 5 Ya genes and 2 Yc genes.

This group also isolated three unique genomic fragments from a rat genomic library using a rat Ya cDNA clone (pGTB38) as a probe, also indicating the presence of multiple genes in this family. The protein products encoded by these genes remain to be elucidated.

Analysis of the nucleotide sequence of these genomic fragments has shown the similarities and differences that exist between them. Comparison of the amino acid sequences encoded by the Ya genomic fragments and equivalent sequences from Yc structural genes has revealed there to be high overall homology in all but exon 3, of the genes (Picket & Lu, 1989). Exon 3 of the Ya genomic fragment shares only a 35% homology with the equivalent Yc amino acid sequence. By contrast, exons 2 and 4 of Ya show 86% and 91% homology with exons 2 and 4 of the Yc subunit. It may be the case that conserved regions in a protein impart similar functional aspects such as glutathione binding domains, while the regions of low homology confer other properties that give each isoenzyme their individual properties such as substrate specificities.

Although Rothkopf et al (1986) have isolated three unique genomic fragments from a rat genomic library and Southern blot analysis has revealed the presence of at least 5 Ya and 2 Yc genes, the precise number of functional genes is unknown. To date, no investigators have characterised a Yc structural gene and all the information regarding homologies has come from cDNA and amino acid sequence data. More data is needed regarding the number and structural characteristics of different genes coding for the Ya, Yc and other class alpha subunits contained within rat genomes.

With regard to the situation in man, cDNAs encoding proteins of this class have been cloned and characterised (Tu & Quian, 1986; Rhoads et al, 1987; Board & Webb, 1987). The human cDNA sequences are 80% homologous with rat liver Ya and Yc cDNAs and the predicted amino acid sequence shows a 75% identity between rat and man. The two cDNA clones pGTH1 (Tu & Quian, 1986) and pGTH2 (Rhoads et al, 1987) have been shown to encode B_1 and B_2 subunits respectively (Hayes et al, 1990). The sequences for B_1 and B_2 although 96% homologous, differ in 9 amino acids

indicating they are the products of two different genes. These structural genes have yet to be isolated and characterised.

(ii) Regulation

The rat liver Ya subunit has been shown to be induced by various xenobiotics such as 3-methylcholanthrene and phenobarbital (Telakowski-Hopkins et al, 1988). The observed increase in enzyme activity has been shown to be due to an increase in translational activity of the Ya mRNA (Pickett et al, 1982a;1982b; Pickett et al, 1984). Work by Ding & Pickett (1985) showed that members of the Ya gene family are transcriptionally activated in rat liver. Subsequently, Telakowski-Hopkins et al (1988) have investigated the mechanisms responsible for this induction. These workers fused various lengths of the 5' flanking region of a rat Ya structural gene to a gene encoding chloramphenical acetyltransferase (CAT) and looked for inducibility of CAT activity by various planar aromatic compounds, such as B-naphthoflavone.

The following features were noted and lead to the proposed mechanism of regulation shown in Fig. 1.4 (Pickett, 1987). Firstly, two regulatory sequences were discovered, one which is required for maximal basal expression and the other which is responsible for inducibility by planar aromatic compounds such as B-naphthoflavone and 3-methylcholanthrene. Secondly, this second regulatory element is only responsive in cells that have a functional dioxin receptor.

The mechanism proposed in Fig. 1.4 is as follows: A polycyclic aromatic hydrocarbon binds to the dioxin receptor forming a complex. This ligand-receptor complex is then translocated to the nucleus where is causes transcriptional activation of the Ya gene by interacting with the regulatory element responsive to polycyclic aromatic hydrocarbons. It could alternatively be the case that the ligand-dioxin receptor complex activates a transacting gene coding for a regulatory protein which in turn regulates expression of the Ya gene by interacting with the regulatory element responsive to polycyclic aromatic hydrocarbons.

Figure 1.4 Proposed models of rat Ya glutathione S-transferase gene expression regulation by polycyclic aromatic hydrocarbons.

There are two proposed mechanisms. (1) A polycyclic hydrocarbon (l) binds to the dioxin receptor (R) forming a complex. This ligand-receptor complex (l-R) is then translocated to the nucleus where it causes transcriptional activation of the Ya gene by reacting with the regulatory element responsive to polycyclic aromatic hydrocarbons. It is proposed that the same mechanism is responsible for P450 induction. (2) Alternatively, the ligand-receptor complex (l-R) activates a gene coding for a regulatory protein (Y) which in turn regulates expression of the Ya gene by interacting with the regulatory element responsive to polycyclic hydrocarbons (Pickett, 1987).

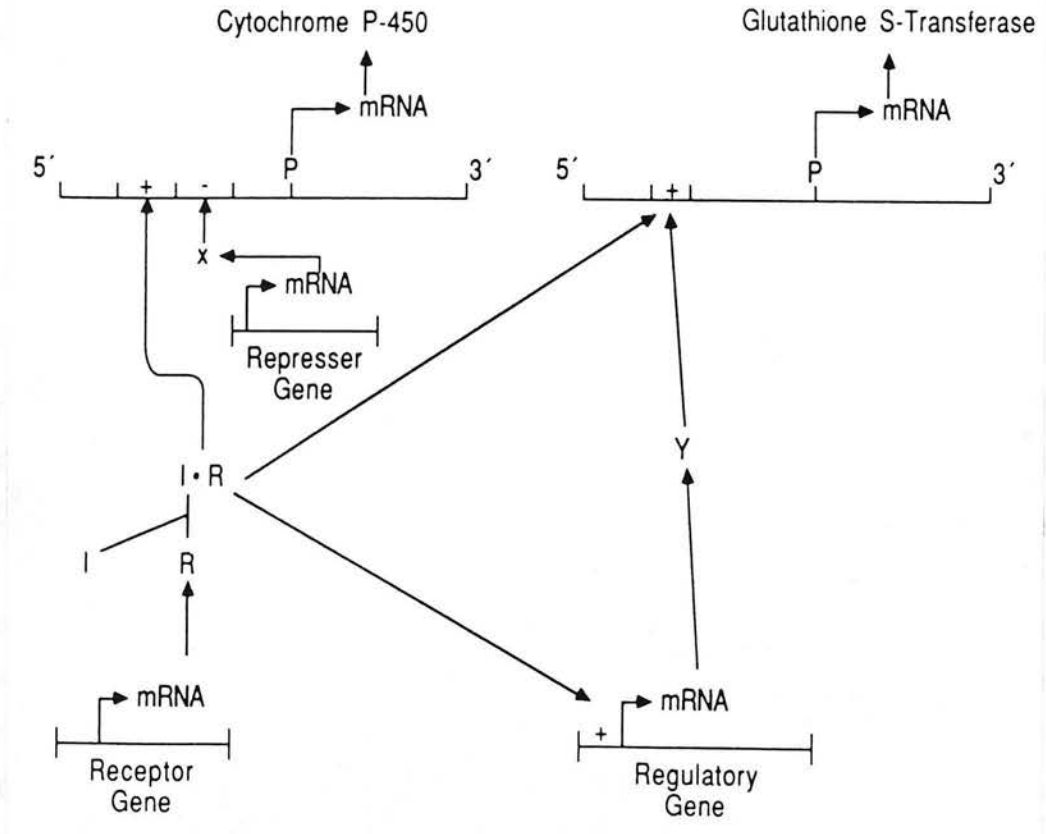


Fig. 10.

To date, it has not been possible to distinguish between the two possibilities. So far, structural features such as those discussed above for rat Ya have not been described for the human alpha class genes and information of this nature is eagerly awaited.

Regulation of the class alpha genes in the mouse has been addressed (McLellan et al, 1989). It has been shown that a mouse liver alpha class subunit which is not normally detectable in liver, is induced by both butylated hydroxyanisole (BHA) and β-naphthoflavone. At the genetic level, Daniel et al (1987), using a mouse Ya subunit linked to a CAT gene, have also shown the presence of two regulatory elements. The situation is the same as in rat, in that one of the regulatory elements is responsible for basal expression and one is responsible for inducible expression by compounds such as 3-methylcholanthrene and β-naphthoflavone. Again, the inducible receptor is only active in the presence of a functional dioxin receptor.

(b) Structure and Regulation of the Class Pi GSTs

(i) Structure

Full length genomic clones for both the rat and human pi class GST have been isolated (Suguoka, et al ,1985; Okuda et al, 1987; Muramatsu et al, 1987; Sakai et al, 1988). Suguoka et al (1985) have isolated the rat YfYf (GST-P) gene from a phage library which they probed with a rat cDNA isolated from a 2-acetylaminofluorene induced rat hepatocellular carcinoma. The rat YfYf gene has been demonstrated to be 3000 base pairs long and consists of 7 exons and 6 introns. These workers also linked the 5' end of the rat YfYf gene to the gene for CAT to allow the investigation of the regulatory DNA sequences in this region. Using this technique, two enhancing elements were found 2500 and 61 base pairs upstream from the translation initiation site. Both enhancers contained a 12-0-tetradecanoyl-phorbol-B-acetate (TPA) responsive element (TRE). Mechanisms for altered gene expression by TPA have been proposed (Nishizuka, 1984; Chiu et al, 1987). This is speculated to involve a signal transduction cascade mechanism triggered by protein kinase C. During this mechanism

specific transacting factors are modified so causing alterations in the expression of the genes with which these factors are active.

A silencing element was also found 400 base pairs upstream from the translation initiation site (CAP site) of the Yf gene. It has been proposed that this may act as some sort of negative regulatory element (Muramatsu et al, 1989). This may function to suppress the expression of YfYf in normal liver cells.

The structure of the human π gene has also been analysed (Cowell et al 1988). The human GST π gene was isolated from a cosmid library constructed with genomic DNA prepared from the acute lymphocytic leukaemia cell line HPB-ALL and the CAT assay was used in order to investigate structures involved in the regulation of this gene. As with the rat sequence, a TRE consensus sequence was noted in the promoter region. However, in contrast to the rat sequence in the 2.2 to 2.5 kilobase upstream region of the human gene, no enhancer sequences were found, only repetitive elements (Cowell et al, 1989). This work indicates that enhancer sequences if present, must be located further than 6kb upstream of the promoter or in a downstream position.

(ii) Regulation

It has been postulated that regulation of the rat liver Yf gene during chemical carcinogenesis takes place by de-repression rather than a transcriptional activation of the gene. Whatever the mechanism, certain factors have been shown to be involved in the expression of the rat Yf gene. Several workers (Power et al, 1987; Li et al, 1988) have shown that both TPA and ras-oncogene expression can increase steady state levels of rat Yf mRNA. The actual mechanism by which this occurs has not yet been elucidated. However, it has been suggested (Cowell et al, 1988) that ras may act through the TRE's of several cellular genes leading to the concomitant induction of various other gene products. This is highly relevant to the tumour specific induction of YfYf seen in rat liver carcinogenesis models, since ras is overexpressed in many tumours.

(c) Structure and Regulation of the Class Mu GSTs

(i) Structure

Again, most of the work in this area has been carried out using the rat model (Ding et al, 1985; Ding et al, 1986; Lai et al, 1986; Lai & Tu, 1986; Abramovitz & Listowsky 1987). To date, at least three rat mu class cDNA clones have been isolated and characterised. They have been referred to as Yb_1, Yb_2 and Yb_3 and are about 80% homologous at the amino acid and nucleotide sequence level in the coding region. The 5' and 3' untranslated regions, however, are divergent. Lai et al, (1986) have carried out Southern blot analysis of rat genomic DNA which indicates the presence of multiple Yb genes.

Three structural genes have been characterised (Tu et al, 1987; Lai et al, 1988) and have been shown to code for the mu class subunits Yb_1, Yb_2 and a Yb_4, the latter of which has not yet been shown to be expressed in rat tissue. A rat Yb_3 subunit has been shown to be expressed in rat brain using a cDNA for this subunit to detect mRNA levels, but the structural gene encoding this has not been isolated. The sequence analysis of the genomic clones Yb_1, Yb_2 and Yb_4 has revealed them to consist of 8 exons separated by 7 introns and containing approximately 5000 base pairs. The nucleotide sequences of exons 3, 4 and 6 are highly conserved and it has been suggested that evolution of these genes occurred due to gene conversion (Lai et al, 1988).

(ii) Regulation

Like the rat class alpha subunits Ya and Yc, the class mu subunits Yb_1 and Yb_2 have also been shown to be elevated by compounds such as phenobarbital and 3-methylcholanthrene (Pickett et al, 1982a; 1982b). This elevation can also be attributed to transcriptional activation of the genes coding for the subunits (Ding & Pickett, 1985). However, the mechanisms by which various xenobiotics cause transcriptional activation of the mu class GST genes remains to be elucidated.

1.2.9 Future Prospects.

It is evident from the preceding sections that there are a range of GST isoenzymes in rat and man whose functions are varied. It is also clear that further molecular characterization of these enzymes is required in order to relate the genes to the protein products.

With the advent of site-directed mutagenesis techniques, more information should become available on the structure/function relationships of the GSTs. Furthermore, the regulation of gene expression and identification of the transacting factors involved will provide more information about the role of GSTs in processes such as carcinogenesis and drug resistance.

1.3.0 THE ROLE OF THE GLUTATHIONE S-TRANSFERASES IN DRUG RESISTANCE

Introduction

As discussed in Section 1.1, the major cause of treatment failure in cancer chemotherapy is the onset of clinical drug resistance to a range of structurally and functionally dissimilar compounds. It is becoming evident that this phenomenon is multifactoral in nature. The role of the glutathione S-transferases in this process is not clear at present but a large body of circumstantial evidence is emerging indicating that GSTs are involved in drug resistance.

1.3.1. Analysis of GST Expression in Human Tumour Samples

Although much of the work carried out on drug resistant cell lines has yielded valuable information, relevance of GST expression to the clinical situation must also be assessed. To this end several investigators have analysed the GST content in various human tumours. The over expression of a number of different GST isoenzymes has been noted in a variety of different tumour types.

The work in human tumour samples has been carried out primarily at the protein level, using enzyme assays and immunological techniques and at the RNA level, using both northern and slot blot analysis. Work by Shea et al (1988) involved measurement of GST activity in 24 human tumour samples, including six colon adenocarcinomas, three renal carcinomas, two melanomas and six breast adenocarcinomas. Purification of the GSTs from the cytosols of these tumours revealed that 70% of the overall GST activity could be attributed to the π subunit.

High levels of the rat class pi subunit Yf are seen in rat hepatic nodules resulting from exposure to carcinogens and it has been postulated that this leads to resistance to other cytotoxic compounds. Therefore, high levels of GST pi may predispose cells in a tumour population to resistance to anticancer drugs.

Other workers have also showed that the GST isoenzyme content in certain tumour tissues is altered in relation to normal tissue from the same organ and individual. Di Ilio et al (1987) have studied GST isoenzyme content in human renal carcinomas. Paired tumour and non-tumour tissue from the same patient were analysed. The overall GST activity, measured using CDNB as a diagnostic substrate, was reduced in the tumour samples. However, analysis of the GST isoenzyme profile using double immunodiffusion techniques, revealed an overall increase in the π subunit content but an overall decrease in the class alpha subunits in tumour versus normal tissue, leading to an overall decrease in total GST activity.

Other work by this group on lung tumours (Di Ilio et al, 1988) has shown GST activity is increased in tumour tissue relative to normal surrounding tissue. Analysis of the isoenzyme profile revealed the increase in GST activity could be attributed to the

class π subunit.

In another study (Soma et al, 1986) it was also found that the GST π enzyme is overexpressed in human hepatic tumours. These workers have suggested that GST π can serve as a hepatic tumour marker. This has also been suggested by other workers.

High levels of GST π were also detected in human malignant melanoma (Mannervik et al, 1987). However, in this study, only two samples were analysed and fibroblasts and naevus samples were used as a normal comparison.

A more comprehensive study of GST π expression in human tumours has been carried out by Moscow et al (1989) who not only looked at a wide variety of tumours but also compared GST π mRNA with P-glycoprotein expression. A total of 170 specimens were analysed and both normal and malignant tissues were compared. In normal tissue, π mRNA levels were low in liver and high in lung, oesophagus and placenta in agreement with other workers. In the tumour samples GST π mRNA levels were found to be low in lymphoma and breast cancer and high in lung and head and neck cancers. In most of the paired tumour and non-tumour specimens, GST was found to be overexpressed in the former. This is particularly true for the colonic carcinomas which is in agreement with Kodate et al (1986) and the stomach samples in which is in agreement with Tsutsume et al (1987).

One criticism that can be made of much of the work carried out in this area is that GST overexpression is rarely correlated with drug treatment status, disease status or clinical outcome. Preliminary results (Moscow et al, 1989) indicate that GST π may be elevated during initial treatment with chemotherapeutic agents since in two patients with relapsed acute lymphoblastic leukaemia, GST π mRNA levels were elevated after treatment, compared to levels detectable before treatment.

Comparison of normal versus tumour tissue is desirable as is the analysis of the actual GST isoenzymes expressed in samples. Correlation of the GST data with other drug resistance markers, as well as several clinical factors, is also required if the

relevance of GSTs to the drug resistant phenotype is to be ascertained.

1.3.2. GST Content of Derived Drug Resistant Cell Lines

As with most of the work concerning P-glycoprotein expression, much of the work on the involvement of GSTs in drug resistance has come from work using cell lines made resistant *in vitro* to certain anticancer drugs. Although informative, obvious extrapolations to the clinical situation cannot be made based on results from these cell line models. Several approaches have been taken in order to study *in vitro* the role of the GSTs in drug resistance:

(1) Selection of a resistant cell line and examination of cross-resistance patterns and various biochemical changes.

(2) Transfection of genes known to code for factors implicated in drug resistance into cells and examination of cross-resistance patterns.

(3) Use of inhibitors of resistance factors to analyse whether drug resistance patterns can be reversed.

Table 1.5 shows drug resistance models where GST involvement has been implicated.

The work in this area is reviewed below. Many drug resistance tumour cell lines have been established from a number of mammalian species. It is possible that mammalian cells from different species may respond differently to treatment with antitumour agents and that expression of the different isoenzymes among species may be subject to different control mechanisms.

The first reports that a protein of molecular weight of about 25,000 daltons was present in a derived drug resistant line and not in the parent line appeared in the early 1980's (Myers & Biedler, 1981; Beck, 1983). This protein was not identified and it is therefore unclear whether this protein represents a GST subunit or not. Since then many reports have appeared in the literature showing that GST activity is elevated in drug resistant cell lines.

Table 1.5 Drug resistant tumour cell line models exhibiting GST overexpression.

CELL LINE/ DRUG RESISTANT SUBLINE	DRUG USED TO DERIVE RESISTANT SUBLINE	FOLD RESISTANCE	DRUGS TO WHICH RESISTANCE OBSERVED	FOLD RESISTANCE	ELEVATION OF GST ACTIVITY	GST SUBUNIT INVOLVED	REFERENCES
MCF-7/MCF-7/Adr (Human breast tumour cell lines)	Adriamycin	192	VP-16 Vincristine Vinblastine Actinomycin D	100 >250 375 175	15-45	GST pi (45-100 fold)	Batist et al (1986) Cowan et al (1986)
MES-SA/MES/DX-5 (Human sarcoma cell lines)	Adriamycin	100	Danorubicin Dactinomycin Vinblastine Vincristine Etoposide Mitomycin	160 1200 105 240 30 10	2.4	No subunit change in class alpha, pi or mu GST.	Lewis (1988)
PE01/PE04 (Human ovarian tumour cell lines)	Chlorambucil		Prednimustine Melphalan Adriamycin Mitoxantrone	8.8 4.2 2.6 2.0	2-3	No subunit change in class alpha, pi or mu GST.	Wolf et al (1987) Lewis et al (1988) Hayward (1987)
CHO-KI/CHO-CHL^r (Chinese Hamster ovary tumour cell lines)	Chlorambucil	24	Melphalan Merchlorethamine	14 34	3	Alpha class (40-fold) YaYc	Robson et al (1986) Robson et al (1987) Lewis et al (1988)
Walker 256-WS/Walker 256-WR (Rat mammary carcinoma cell lines)	Chlorambucil	15-27	Nitrogen mustard Phosphoramide mustard Cis-platin Mitomycin C	20 12 23 20	2.4	Alpha class (Yc)	Tew & Wang (1982) Tew et al (1985) Wang & Tew (1985) Buller et al (1987)
9L cells (Rat gliosarcoma cell lines)	BCNU	3-4	2-[3-(chloroethyl)-3-nitrosoureido]-D-dioxygluco-pyranose		2.5*	Mu class GST	Evans et al (1987) Smith et al (1989)
P388/P388/Adr (Murine leukemia cell line)	Adriamycin	100	Actinomycin D Daunorubicin Mitoxantrone Colchicine Etoposide	18 16 11 12 6	1.4	GST pi	Deffie et al (1988)

* Denotes fold decrease in overall GST activity.

In a large number of cases, however, overall GST activity has been used as a measure of overexpression. This may sometimes be misleading as has been shown by Di Ilio et al (1987), who reported that in renal carcinomas the π subunit was up-regulated while the class alpha subunits were down regulated, leading to an overall decrease in total GST activity. Changes in individual isoenzyme content may have greater implications for drug resistance than a change in total GST activity.

This has been shown to be the case for a rat brain tumour cell line resistant to BCNU termed 9L (parent line) and 9L-2 (BCNU resistant line). Smith et al (1989) found that although the overall GST activity of 9L-2 had decreased by about 50%, there was an increase in a class mu enzyme and a decrease in the class pi enzyme. It was suggested that this up-regulation in the class mu enzyme was contributing to BCNU resistance. Further evidence for this hypothesis came from the use of GST inhibitors to sensitise the 9L-2 cells to BCNU.

Others, working on a rat mammary carcinoma cell line resistant to chlorambucil (Wang & Tew 1985; Buller et al, 1987; Clapper & Tew, 1989) have shown overexpression of an alpha class subunit in the resistant subline. There was a 4-fold increase in GST activity detected in the resistant line (WR) and it was shown that during selection with chlorambucil, increasing the concentration of the drug led to an concomitant rise in GST activity up to a maximum of 4-fold. As with all of these generated models, the authors were unable to say whether the overexpression of the class alpha subunit had occurred by selection of a subpopulation of cells or by an induction mechanism due to drug treatment.

Further to the work illustrating over-expression of certain cytosolic GSTs, the subcellular fractions of the chlorambucil resistant line were also analysed. The microsomal fraction was subjected to S-hexyl-glutathione affinity purification. Two proteins of molecular weight 25.7 and 29.0 kilodaltons were isolated from WR and antisera raised against total liver cytosolic GST cross-reacted with these proteins. The 29kD protein was not found in the chlorambucil sensitive parent line and was immunologically distinct from the known 14kD microsomal GST. These workers suggested that a novel microsomal GST was present in WR and that this may be contributing to resistance to chlorambucil. However, it is possible that the 29kd protein

identified in the microsomal fraction of WR represents contamination by a cytosolic GST.

The pi class enzymes have also been implicated in drug resistance as well as members of the other two GST classes discussed above. Gupta et al (1989) have derived human plasma lines resistant to melphalan. Upon injection of these lines into mice, plasmacytomas were formed. The GSTs were purified from these plasmacytomas and subjected to western blot analysis. A moderate overexpression of the pi class enzyme was seen. The functional characteristics of the overexpressed class pi enzyme were found to differ to those recorded for the class pi enzyme in rat, mouse and man. Whether these workers have isolated a novel class pi enzyme remains to be established.

Other rodent models have been examined where overexpression of more than one class of GST isoenzyme is reported (Robson et al, 1986; Robson et al, 1987; Singh et al, 1989), In a Chinese hamster ovary cell line 20-fold resistant to chlorambucil (CHO-CHL^r) but showing little cross-resistance to other drugs except mechlorethamine and melphalan, a cytosolic protein of molecular weight 25kd was found to be overexpressed (Robson et al, 1986). Upon further analysis, this resistant line was found to have reduced levels of DNA cross-links but an apparent unchanged DNA repair capacity. The CHO-CHL^r showed a 3-fold increase in overall GST activity, provided by a 2-fold increase in the class pi enzyme and a 5-fold increase in class alpha enzyme activity (Robson et al, 1987). The overexpressed enzymes were purified from the cytosolic fraction of the CHO-CHL^r and subjected to western blot analysis (Lewis et al, 1988). There was seen to be a 40-fold overexpression of a class alpha subunit that cross-reacted with anti-rat Yc antisera. A pi class subunit and another class alpha (Ya) were also shown to be overexpressed. The mechanisms of overexpression of the class alpha subunits were examined. Northern blot analysis revealed significantly higher levels of the mRNA coding for these subunits and Southern blot analysis revealed a 4- to 8-fold increase in gene copy number of the class alpha subunits in the CHO-CHL^r cell line.

Concomitant increases in class alpha and class pi subunits have been reported in

a murine leukaemic cell line P388, resistant to adriamycin (Sing et al, 1989). These workers however, did not use immunological techniques to analyse the overexpressed subunits but employed the diagnostic substrates cumene hydroperoxide and ethacrynic acid. These workers suggested that the class alpha GST may contribute to resistance by enhancing free-radical detoxification, since the generation of oxyradicals by the redox cycling of the quinone moiety of adriamycin is believed to contribute to the antitumour activity of this drug. This view is also supported by other workers (Kramer et al, 1988).

Other groups also working on derived adriamycin resistant cell lines have found increases in GST activity, up to 45-fold (Batist et al, 1986). These workers were investigating the human breast tumour cell line, MCF 7 which expresses intrinsically low GST activity. The adriamycin resistant subline MCF 7/ADR, however, has been shown to express a class pi enzyme which is not seen in the parent line. The class pi enzyme was purified from MCF 7/ADR^r and subjected to further analysis.

Batist and co-workers (1986) reported that this anionic GST, which is homologous to the class pi subunit overexpressed in rat preneoplastic modules, also had high levels of intrinsic peroxidase activity. This finding has not been reproduced by others and this raises the question of the purity of the GST these workers isolated. A cDNA clone of this anionic GST has subsequently been isolated (Moscow et al, 1989) and the nucleotide sequence in the protein coding region is essentially identical to the sequence reported by Kano et al (1987).

Much of the work discussed above has detailed one type of change that may have occurred in a drug resistant cell line. However, the emerging picture from the clinical data is that drug resistance is a multifactoral process involving P-glycoprotein expression, DNA repair and topoisomerase II. Factors may of course predominate depending on the model studied.

In a murine lymphoblast line resistant to hydrolysed benzoquinone mustard (Begleiter et al, 1988) several factors associated with drug resistance were analysed. Drug uptake was found to be lower in the resistant cell line. Of the enzymes analysed, superoxide dismutase, catalase, glutathione peroxidase, glutathione S-transferase and

DT-diaphorase were all increased. The individual GST isoenzyme changes were not investigated. These overexpressed enzymes may contribute to resistance by inactivating free radicals formed by action of the drug.

Deffie et al (1988) studied an adriamycin resistant murine leukaemic cell line and showed there to be no difference in drug efflux, indicating that an active drug extrusion pump was not operational. However, there was an increase (2-fold) in P-glycoprotein expression as detected by northern and western blot analysis. Total GST activity was estimated to be increased by 1.44-fold and the levels of class pi mRNA and protein was shown to be elevated.

As well as the multifactoral nature of drug resistance, another aspect of this phenomenon that needs to be addressed is the relevance of these *in vitro* models to the *in vivo* situation. In an attempt to study this, a number of workers have generated cell lines from surgically removed tumours in order to investigate whether the characteristic drug resistance of tumours is also evident in the derived cell lines and if so, what factors may be involved (Carmichael et al, 1988).

Non-small cell and small cell lung tumour cell lines were established from patients with the corresponding disease (Carmichael et al,1988). The chemosensitivity patterns of these cell lines to a variety of anticancer drugs were similar to those observed clinically. Certain drug detoxification enzymes were analysed, including GST. The the non-small cell lines demonstrated an overall increase in GST activity of about 2.5-fold when compared to the small cell lines. This correlates with the patterns of drug resistance seen in the two clinical tumour types, in that small cell lung cancer is usually drug sensitive upon presentation but develops resistance in response to treatment whereas non-small cell lung cancer is refractory to treatment and so intrinsically resistant (Bergsagel & Feld, 1986; Ruckdeschel et al, 1986). In this study, there was no correlation with treated and non-treated patients. The individual GST isoenzyme profiles were not examined, and as mentioned previously a measurement of overall GST activity may mask individual isoenzyme changes (Di Ilio et al, 1987).

A review of some of the major work in this field would not be complete without reference to work on derived drug resistant cell lines where GST overexpression is not noted. Bellamy et al (1989) showed that in a human myeloma cell line resistant to

adriamycin, no overall difference in GST activity was seen. Using a cDNA for the π subunit no increase in mRNA levels was noted. Using cumeme hydroperoxide as a diagnostic substrate, no increase in the class alpha enzyme activity was observed. However, these workers offered no explanation as to the possible mechanisms of resistance operational in this cell line.

Work by Yusa et al (1988) showed that a colchicine resistant MCF 7 cell line exhibited a 70-fold increase in overall GST activity. A subline that reverted to being colchicine sensitive, however, still showed the overexpression of GST activity. The π subunit was shown to be responsible for the increased GST activity in the resistant and revertant line. These results prompted the authors to conclude that GSTs are not associated with the development of drug resistance. However, in order to analyse the role of GSTs in drug resistance, all the available data needs to be considered.

The two major criticisms that arise from the work reviewed above are as follows. Firstly, in every case it is impossible to say whether or not the GSTs confer resistance or are overexpressed non-specifically as a result of the many changes that are presumed to occur during the resistance phenomenon. Secondly, there are few reports of anticancer drug-glutathione conjugates formed as a result of GST catalysis and little available evidence as yet that different anticancer drugs can act as GST substrates.

In order to answer the question as to whether GSTs can actually confer resistance, several workers have cloned GSTs into various vectors and used these to transfect different cell lines (Manoharan et al, 1987; Black et al, 1990).

Puchalski et al (1989) transfected mouse cells with expressions vectors that contained either Ya, Yb_1 or π subunits and which were driven by the α collagen promoter. The transfected cells were then subjected to colony forming assays in order to assess the sensitivity of wild type and transfected cells to a variety of anticancer drugs. The cell lines expressing Ya were 1.5, 2.5 and 2.9-fold resistant to chlorambucil.

Other workers have cloned cDNAs for human B_1 and π subunits into the yeast expression vector pMA56 and expressed these in *S. cerevisiae* (Black et al, 1990).

The sensitivity of the wild type and transfected strains to a variety of drugs were tested. Both subunits appear to afford protection against chlorambucil and adriamycin. In addition to this, the B_1 subunit afforded protection against CDNB and cumene hydroperoxide. This work suggests that the GSTs do indeed have the ability to confer drug resistance and has implications for the drug resistant cell line models reviewed earlier. That is, it is possible that the GST may be responsible, or partially responsible, for the drug resistance seen in these cell lines. However, as already highlighted in Section 1.1., it is probable that more than one factor is involved in the multidrug resistant phenotype and the relative importance of the GST in this phenomenon needs to be assessed. The question as to whether anticancer drugs can act as substrates for the GSTs has also been addressed. Dulik et al (1986) have isolated melphalan-glutathione conjugates and have demonstrated that adduct formation is catalysed by both microsomal and cytosolic GSTs. However, few workers with drug resistant cell line models have shown that drug-glutathione conjugates can be isolated from these drug resistant cell lines.

1.3.3 CONCLUSION

As can be seen, drug resistance to a wide range of anticancer drugs can arise by a variety of different mechanisms. The relative role of the GSTs in this phenomenon needs clarification. Since these enzymes have been shown to fulfill a number of different and varied functions, it is quite feasible that they could detoxify certain anticancer drugs so reducing their cellular concentration and hence their harmful cytotoxic effects. Further data regarding the ability of the different isoenzymes to detoxify anticancer drugs and confer the multidrug resistant phenotype on tumours cells is awaited.

1.4.0 OBJECTIVES OF THE STUDY

The major aim of this project is to study the role of the glutathione S-transferases in resistance to cytotoxic compounds. In order to do this , known GST substrates will be used to generate resistant tumour cell lines. Whether directed overexpression of GST subunits can be achieved, using such a model, will be investigated. The cross-resistance patterns of these models to cytotoxic compounds and anticancer drugs will be analysed in order to ascertain whether resistance to structurally and functionally distinct compounds can be achieved potentially through only one mechanism, in this case GST catalysed detoxification.

A secondary aim of this project is to investigate whether the treatment of cells with cytotoxic compounds can elicit a stress response and if so, whether certain GSTs may function as stress response proteins.

The expression of the pi class GST in clinical leukaemia samples will also be investigated and correlated with P-glycoprotein overexpression in the same leukaemia samples.

CHAPTER TWO

MATERIALS AND METHODS

2.1.0 CELL CULTURE

2.1.1 Growing and Routine Maintenance of Human Tumour Cell Lines

All cell lines used during this work were taken from laboratory stocks of the lines that had been stored in liquid nitrogen until required. The cell lines used were MCF 7, PE04, NCI H322 and HT29 which were human breast carcinoma, ovarian adenocarcinoma, lung carcinoma and colonic carcinoma respectively. The cell lines were all grown in RPMI 1640 media which was supplemented with 10% (v/v) foetal calf serum, streptomycin (100μg/ml) and penicillin (100 IU/ml). Insulin (2.5mg/ml) was also supplemented to the media in which the PE04 cell line was grown.

The cell lines were cultured at 37°C in an atmosphere of 5% CO_2 at 100% humidity. Before commencing experiments with the various cell lines, frozen stocks at one particular passage were obtained for each line.

Cells were cultured for no more than eight passages. After eight passages, the cells were discarded and fresh cryopreserved cells from the frozen stocks were utilised. This was to avoid, as far as possible, phenotypic and genotypic changes that have been shown to occur in cells over prolonged periods of time in culture.

To cryopreserve cells at the same passage, 50 to 100 x 10^6 cells/ml were aliquoted into 2ml of freezing mix which consisted of 90% (v/v) newborn calf serum and 10% (v/v) dimethyl sulphoxide. Cells were first frozen at -70°C for 24 hours and then transferred to liquid nitrogen. When these cryopreserved cells were required, they were thawed rapidly in a water bath at 37°C, washed twice in the appropriate medium to remove the dimethyl sulphoxide and transferred to a 1 x 25 cm^2 flask (Nunc) in 5-10 ml of the appropriate media.

The cells were allowed to settle overnight and refed with fresh medium the next day to remove any cell debris. Cultures of cells were fed every 2-3 days with the appropriate medium and sub-cultured (passaged) as necessary. This involved harvesting the cells(section 2.1.5) with sterile 0.1% (w/v) trypsin and 0.001% (w/v) versene (EDTA) mixed in a ratio of 1:1 and dividing the resultant cell suspension into equal volumes before seeding it into new flasks.

This procedure was to maintain cells in logarithmic growth and avoid the cells becoming over confluent. Each time this procedure was carried out, one passage was added to the passage at the start of the culture.

2.1.2 Mycoplasma Testing

Periodic assays for mycoplasma were carried out routinely by the MRC Clinical and Population Cytogenetics Unit, Western General Hospital, Edinburgh and were always found to be negative. The assays involved growth of mycoplasma on defined agar plates and also a fluorescence test.

2.1.3 Derivation of an Ethacrynic Acid Resistant MCF 7 Cell Line

8 x 75 cm^2 flasks (Nunc) were seeded with 10^7 cells. Four flasks were maintained as described in Section 2.1.1. The other four flasks were dosed with ethacrynic acid at concentrations of 5μM, 10μM, 25μM and 50μM. The cells were observed and photographed daily and dosed every two days. The derivation process is shown in Fig. 3.05 (chapter 3).

Cells were dosed by tipping off the media containing ethacrynic acid, in which the cells were growing, and washing the cells three times with PBS (140mM NaCl; 2.7M KCl; 8mM sodium phosphate, pH 7.4). 20ml of fresh medium containing the appropriate concentration of ethacrynic acid made from a stock solution was then added. The stock solution of ethacrynic acid was freshly prepared every time the cells were dosed.

The stock solution was made by weighing out the appropriate amount of ethacrynic acid on a Mettler A E 160 electronic balance, dissolving this in dimethyl sulphoxide and diluting this into serum free medium. This was then filter sterilised using a sterile 10 ml syringe (Becton Dickson) and a 0.22μM filter (Millipore) before adding to the culture

medium.

To give a final ethacrynic acid concentration of 25μM, 0.5mls of a 10^{-3}M stock solution of ethacrynic acid was added to 19.5 ml of culture medium. It was cells dosed at this ethacrynic acid concentration that were used in the derivation of a drug resistant subline since other drug concentrations either had no observable effects on cells (5 and 10μM) or killed 100% of the cells (50μM).

MCF 7 cells were cultured in the presence of ethacrynic acid for about two months. Dosing cells at 25μM of ethacrynic acid also meant that dimethyl sulphoxide was present in the culture medium at a concentration of 0.025% (v/v). To rule out any changes that may have resulted from this, control cells were also cultured in the presence of 0.025% (v/v) dimethyl sulphoxide.

2.1.4 Derivation of a CDNB resistant NCI H322 Cell Line

8 x 75cm^2 flasks (Nunc) were seeded with 10^7 NCI H322 cells. Four flasks were maintained as described in Section 2.1.1. The other four flasks were dosed with CDNB at a concentration of 10μM or 25μM. The higher dose of CDNB proved to be toxic at this stage of the derivation process so cells dosed at 10μM were used to derive a resistant cell line.

There was a stepwise increase in the concentration of CDNB up to a maximum of 25μM over an eight month period. This is represented in Figure 3.05 (chapter 3).

With each increase in CDNB concentration there was a certain amount of cell death. However, at each stage small colonies of cells remained which eventually became confluent even in the presence of the CDNB. Frozen stocks of cells were obtained at every stage of the derivation process.

Cells were dosed every two days. This was done by tipping off the media containing CDNB, in which the cells were cultured and washing the cells three times with PBS. 20ml of fresh media containing the appropriate dose of CDNB made from a stock solution of CDNB was then added. The stock solution of CDNB was freshly prepared each time the cells were dosed. The stock solution of CDNB was made by weighing the appropriate amount of CDNB on a Mettler A C 160 electronic balance and dissolving this in dimethyl sulphoxide. This was then diluted in serum free media and filter sterilised using a

10ml syringe (Becton Dickson) and a 0.22μM filter (Millipore) before adding to the medium.

For example, to give a final CDNB concentration of 25μM, 0.5mls of a 10^{-3}M stock solution was added to 19.5ml of culture medium. Dosing cells at this concentration of CDNB also meant that dimethyl sulphoxide was present in the culture medium at a concentration of 0.025% (v/v). To rule out changes that may have resulted from this, control cells were also dosed with 0.025% (v/v) of dimethyl sulphoxide.

After eight months, resistance to CDNB was analysed using the MTT assay (Section 2.1.6). At this stage of the derivation process, some cells were maintained in the presence of CDNB and some were maintained in the absence of CDNB.

2.1.5 Harvesting and Counting of Viable Cells

Cells were harvested with 0.1% (w/v) trypsin and 0.001% (w/v) versene (EDTA) in a ratio of 1:1. The media was tipped off the cells which were then washed three times in PBS. The trypsin/versene mix was added in a sufficient volume to just cover the cells and the flask was incubated at 37° until cells were seen to begin lifting off. The flask was shaken gently and the resultant cell suspension was washed three times with medium before suspending the cells in 10 mls of culture medium

The number of viable cells was assessed using a hemocytometer (Scientific Furnishings). 100μl of cell suspension was mixed with 100μl of 0.5% (w/v) nigrosin. 10μl of this suspension was loaded onto a pre-prepared hemocytometer and the cells were observed under phase contrast microscopy. Any dark blue cells were non-viable since viable cells do not take up the nigrosin dye. Viable cells were counted and the concentration of cells per ml was calculated.

2.1.6 Assessment of Cell Sensitivity to Cytotoxic Compounds using the MTT Assay

Cells were harvested and counted as described in Section 2.1.5. A suspension of 2.8×10^4 cells was then prepared by adding the appropriate volume of cells to an appropriate volume of medium. 0.18 ml of this 2.8×10^4 cells/ml suspension was added to

all wells of a 96-well plate (Costar) apart from the wells on the perimeter of the plate. 200µl of medium only was added to the wells on the perimeter of the plate.

The final number of cells in each well was 5 x 10^3 cells per well. The plates were then incubated overnight at 37°C, 5% Co_2 and 100% humidity to permit cell attachment. The following day the appropriate drug dilutions were prepared and 0.02ml of each drug concentration was added to three consecutive wells of the plate to allow triplicate determinations of cell viability at each drug concentration. Six wells were left free of drug for determination of 100% cell viability and if drugs had been dissolved in dimethyl sulphoxide then the appropriate concentration of DMSO was added to six wells and this was used to assess 100% cell viability. The drug was left on the cells for 5 days after which time 50µl of MTT (2mg/ml) was added to each well and the plate left for four hours. After this time, the liquid was carefully aspirated from each well taking care not to disturb the crystals that had formed.

50µl of DMSO was added to the wells to dissolve the crystals and the absorbance of the resultant solution was determined at 540nm using a 2550 EIA reader (Biorad Laboratories). Rows AI to HI were used to zero the machine and the values recorded for the wells with no drug or an appropriate concentration of DMSO, were used as a measure of 100% cell viability. Percentage viability of cells dosed with different drug concentrations was calculated relative to the value for 100% cell viability. Mean and standard deviation were calculated for triplicate determinations of cell viability. The LD_{50} for a particular drug was calculated from the plot of cell viability vs. drug concentration.

2.2.0 PREPARATION OF CELLULAR FRACTIONS

2.2.1. Preparation of Cell Cytosol Samples

Cultures of subconfluent cells were harvested from 75cm^2 flasks (Nunc) with 0.1% (w/v) trypsin and 0.001% (w/v) versene (EDTA) mixed in a 1:1 ratio. The cells were washed three times in medium and resuspended in 250µl of sterile distilled water. Cell viability was determined to be greater than 95% as assessed by nigrosin dye exclusion. This cell suspension was subjected to three 5-second pulses from an MSE Soniprep 150 at maximal power in order to lyse the cells. Each sample was cooled for 30 seconds at 0°

between each burst from the sonicator. 250µl of KCl phosphate (40mM KPO_4, 4.6% (w/v) KCl, 0.4mM EDTA, pH 7.7) was added to each sample before spinning at 13,000 rpm (Eppendorf 5414 centrifuge). The supernatant was decanted leaving behind the pellet. Both fractions were stored at -70°C.

2.2.2 Preparation of Cell Membrane Fractions

The pellet resulting from the procedure described in Section 2.2.1 was used as a crude membrane preparation.

2.2.3 Preparation of Cell Nuclei Fractions

The method described below was followed (A. Bartoszek, personnal communication). This method was loosely based on the method of Taylor et al, 1979 except that ficoll was used instead of sucrose to create a gradient. Cells from a 175cm^2 flask (Nunc) were harvested with 0.1% (w/v) trypsin and 0.001% (w/v) versene, mixed in a ratio of 1:1. The cells were pelleted, then suspended in 1.5ml of buffer H and homogenised gently in a glass homogeniser with fifteen strokes of the plunger. The homogenate was transferred to an eppendorf and spun at 13,000 rpm for 5 minutes (Eppendorf 5414 centrifuge). The pellet that was formed was mixed with 0.5ml of buffer H. This was then mixed with 1.5ml of 20% ficoll (w/v) in buffer H and layered onto 4ml of 20% ficoll (w/v) in buffer H. The nuclei were spun through the ficoll at 4° for 15 minutes at a speed of 5,000 rpm in a Sorvall RT 6000 (DuPont).

The supernatant was gently aspirated off, taking care not to disturb the pellet. The pellet was rinsed with buffer H and suspended in 100µl of PBS. 1-2µl of the nuclei suspension were loaded onto a prepared hemocytometer to verify the presence of intact nuclei. The nuclei were stored at -70°C.

Buffer H

10mM NaH_2PO_4; pH 8.0

2mM Mg Cl_2

2mM DTT

1mM EDTA

2.3.0 Protein Estimation

The method of Lowry et al (1951) was used to determine the protein content of the samples. Bovine serum albumin fraction IV was used as a standard and the following reagents were used to carry out the assay:

A) 70mM $NaCO_3$ 10 H_2O: 40mM NaOH

B) 40mM $CuSO_4$ $5H_2O$

C) 71mM NaK tartrate

D) Stock Folin Ciocalteau reagent diluted 1:1 (v/v) with distilled water

E) Alkaline copper solution which was made up fresh by adding 0.5ml of both solutions B and C and making the volume up to 50mls with solution A.

The following technique was used for cytosol, crude membrane and nuclei preparations. The samples were diluted 1:200 (v/v) with 0.1 m NaOH to give a final volume of 1ml. 5ml of solution E was added to the samples which were then vortexed and left for 10 minutes.

1ml of solution D was then added, the samples vortexed again and left for 45 minutes. Standard solutions of bovine serum albumin at 0, 25, 50, 80, 100, 120, 150 and 200 µg/ml were also treated in exactly the same way.

The absorbance of the standard solution was measured at 600nm on a Schimadzu UV 160 spectrophotometer and a standard curve generated. The amount of protein in each sample was then measured and determined at 600 nm.

A new standard curve was constructed each time a protein determination was carried out. Occasionally sample volume or protein concentration was low and so a microassay was used to assess the protein concentration of such samples. The above method was carried out

but the volume of samples and reagents used was reduced by a factor of 5 and the samples were diluted 1: 50.

Standard protein concentrations were carried out in duplicate and sample protein determinations were carried out in triplicate.

2.4.0 SDS Polyacrylamide Gel Electrophoresis

The method used by Hayes et al (1979) based on the discontinuous buffer system developed by Laemelli (1970) was closely followed. A gel sandwich was prepared by inserting clean spacers between two clean plates of dimensions (12cm x 12cm) and (12cm x 14cm) and inserting the sandwich formed between two clamps from the Biorad Protean 1 apparatus (Biorad Laboratories, UK). This was then positioned on the base of the apparatus so that the glass plate sandwich was sealed on three sides ready for casting the gel. This was performed as follows:

Firstly, the separating gel consisting of 14.8 mls of a solution containing 0.32% (w/v) N,N'-methylene bisacrylamide and 30% (w/v) acrylamide, 9.25 mls of stacking gel buffer (1.5m tris HCl, 0.5% [w/v]; SDS, pH 8.8) and 11.0 mls of distilled water, was prepared. This solution was mixed thoroughly and 2mls of a 1% (w/v) solution of ammonium persulphate followed by 20µl of N,N,N,N'-tetramethylene diamine (TEMED) was added in order to polymerise the gel. The gel solution was quickly mixed, poured to a height of 12 cm into the glass plate sandwich, overlayed with distilled water and left for 30 minutes to allow polymerisation to occur. The overlay was then tipped off and a clean 15, 20 or 25 track comb was inserted between the two glass plates.

The stacking gel was then cast. This consisted of 1.5 mls of the acrylamide/bis-acrylamide solution used for the separating gel, 2.5 mls of stacking gel buffer (0.5M tris HCl, 0.5% [w/v] SDS, pH 6.8) and 5.7 mls of distilled water. This solution was mixed before adding 0.3 mls of 1% ammonium persulphate and 10µl TEMED. The stacking gel was then cast taking care to avoid formation of bubbles. The gel was left for 30 minutes to allow polymerisation to occur and then the comb was gently removed.

The gel was then slotted onto the upper reservoir of the Biorad Protean 1 kit which also contained the cooling system. 1 litre of electrode buffer (0.52M tris, 0.53M glycine, 35mM SDS, pH 7.3) diluted 1:10 (v/v) with distilled water, was added to the lower

reservoir. The upper reservoir was filled with electrode buffer diluted 1:10 with distilled water and the wells of the stacking gel were flushed to remove any debris.

The protein samples were prepared by diluting them with distilled water and adding boiling mix (10% (v/v) stacking gel buffer, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 10% (v/v) glycerol and 0.005% (w/v) bromophenol blue) at a ratio of 1:1 to give a final protein concentration of 1 mg/ml. Samples treated in this way were heated to 100°C for 5 minutes prior to loading in order to denature and solubilise the proteins. The samples were loaded with a Hamilton syringe (Hamilton, Bonadoz, Switzerland). Standard purified GST proteins were treated in the same way as the samples.

The upper reservoir was inserted into the tank containing the lower reservoir. The cooling system was switched on to provide a steady flow of cold water and the lid of the apparatus containing the electrodes was positioned. The current was maintained at 20mA per gel as the dye front ran to the bottom of the stacking gel and was increased to 30mA per gel as the dye front ran to 1cm above the bottom of the separating gel.

If it was necessary to visualise the protein pattern after electrophoresis, the gels were removed from the glass plates by prising the plates apart. The stacking gel was removed and the separating gel was immersed in 0.25% (w/v) solution of Coomasie Brilliant Blue R in water/methanol/acetic acid solution in a ratio of 5:50:7 by volume and left for 1 hour. To destain the gel, it was transferred to a water/methanol/acetic acid solution in a ratio of 88:5:7 by volume. After several changes of destain solution, the protein pattern was viewed on a light box.

2.5.0 Western Blotting

Western blotting was carried out according to the method of Towbin et al (1979) and modified according to Adams et al (1985). The method of SDS/PAGE as described in Section 2.4.0 was used to resolve the polypeptides in the samples under analysis. The gels were treated as follows.

After removal of the stacking gel, the separating gel was trimmed by cutting along the dye front. If more than one gel was being analysed, small incisions were made at the bottom right or left hand corners of the gel to permit identification of the different gels.

The transblot cassette was pre-soaked in the electroblotting buffer (20mM Na_2HPO_4, 20% [v/v] methanol). The gel was orientated in the apparatus so that it was placed on top of a piece of 0.45 μM nitrocellulose which had been cut to the same size as the gel. This was sandwiched between two pieces of scotchbrite pad. The pad was placed into the transblot tank with the nitrocellulose nearest the anode. The tank was filled with electroblotting buffer and the gel blotted overnight at 0.25A.

After transfer, the transblot kit was disassembled and the nitrocellulose removed. The nitrocellulose was washed for two 10 minute periods in TBST (50mM tris/HCl, pH 7.9, 0.15M NaCl, 0.05% [v/v] Tween 20) before blocking in 3% (w/v) low fat milk in TBST, for one hour.

After this procedure, the nitrocellulose filters were given four 10 minute washes in TBST before incubating with the specific GST antisera. The antisera was diluted 1:500 (v/v) in TBST.

Filters were given four 15 minute washes in TBST and then incubated for one hour in goat anti-rabbit IgG antibody conjugated to horseraddish peroxidase (Scottish Antibody Production Unit, Glasgow, Scotland) diluted 1:1000 (v/v) in TBST. Following four 15 minute washes in TBST, the filters were incubated at room temperature in 200 mls TBS containing 0.1 M 4-chloro-1-naphthol and 8 μl of 30% (v/v) hydrogen peroxide, in order to visualise the bands.

Subsequent to this, the filters were washed in distilled water before incubating them in 50 mls of TBST containing 0.19mBq ^{125}I protein A for 1 hour. The filters were washed in several changes of TBST until the counts on the filter approached background levels as ascertained using a Geiger counter. After blotting dry, the filters were autoradiographed for differing time periods using Kodak Xar5 film.

2.6.0 Isolation and analysis of nucleic acids

2.6.1 Isolation of DNA

1 x 175cm^2 flasks (Nunc) of cell lines were harvested for DNA isolation using the method described in Section 2.1.5. The cells were washed three times in PBS and then suspended in 5 mls of lysis buffer (0.5% [w/v] SDS, 150mM NaCl, 10mM tris HCl, 10mM

EDTA, pH 7.5). The cells were shaken well and RNAse at a concentration of 10 µl/ml was added from a stock (10mg/ml) solution.

This solution was incubated for 1 hour at 37°C before adding pronase at a concentration of 50 µl/ml from a stock 5mg/ml solution and incubating for a further 4 hours at 37°C. At this stage 5ml of TE (10mM Tris, 1mM EDTA, pH 8.0) buffered phenol was added and the solution shaken vigorously for 2 minutes before spinning at 3000 rpm in a Sorvall RT 6000 (Du Pont) at 4°C for 10 minutes. The aqueous layer was removed and an equal volume of TE buffered phenol/chloroform mixture in a v/v ratio of 1:1 was added to it. The mixture was shaken and spun as above and the aqueous phase removed and extracted with chloroform.

The aqueous phase formed after this last extraction was mixed with a half volume of 7.5 M ammonium acetate and 2 volumes of absolute alcohol. The DNA was allowed to precipitate overnight at 4°C and then spooled out using a pasteur pipette. It was air dried before dissolving in distilled water.

2.6.2 Isolation of RNA

The method according to Birboim (1988) was carried out on 175cm^2 flasks (Nunc) of confluent cells. In order to minimise degradation of RNA by contaminating nucleases, plastic and glassware used for the procedure described below was treated with distilled water containing 0.1% DEPC for 12 hours before autoclaving the glassware and using the plastic.

The media was removed and the cells washed with cold PBS before 5 ml of RES was added. The flask was swirled gently for 2 minutes until the monolayer of cells lifted off. The resultant lysate was transferred to a falcon tube and sonicated at low power on an MSE Soniprep 150 for 5-10 seconds before adding 450 µl of pronase (1mg/ml stock) and incubating at 50°C for 30 minutes.

The mixture was then cooled to room temperature and 500 µl of 2M sodium acetate and 17.5 ml of ethanol at -20°C were added. The mixture was left for 20 minutes

at -20°C before spinning at 10,000 rpm for 10 minutes in a Sorvall RC-5B (Du Pont) at 0°C. The pellet that was formed was dissolved in RES and extracted with chloroform. The aqueous phase was precipitated by incubating it with 15 µl of 2M acetic acid and 1 ml LiCl/ethanol overnight at 0°C before spinning at 13,00 rpm (eppendorf 5414 centrifuge) for 2 minutes. The supernatant was removed and the pellet dissolved in 500 µl of CCS.

RES

0.5M LiCl

1.0M urea

0.25% SDS (w/v)

0.02% Sodium citrate

25.0mM CDTA

Final pH 6.8.

LiCl/ethanol

3 volumes 5m LiCl

2 volumes ethanol

CCS

1mM Sodium citrate

1mM CDTA

0.1% (w/v) SDS

pH 6.8

2.6.3 Spectrophotometric Quantification of DNA and RNA

DNA and RNA concentrations were measured spectrophotometrically at 260 and 280 nm. Concentrations of nucleic acid given below correspond to a change of 1 absorbance unit.

1 absorbance unit at 260nm = 50mg/ml for double stranded DNA

= 40mg/ml for single stranded DNA and RNA.

The ratio between absorbance at 260 and 280 nm provides an estimate of the purity of the preparation. For a pure DNA preparation the OD_{260}/OD_{280} ratio should be 1.8, and for RNA and the ratio should be 2.0.

2.6.4 Southern Blot Analysis

The DNA isolated by the method described in Section 2.7.0 was restricted using the endonucleases EcoRI, BamHI, HindIII and PstI. 10 µg of DNA was restricted in a total volume of 50 µl. To the appropriate volume of DNA was added 3 µl of the required restriction enzyme, 5 µl of the appropriate buffer, 5 µl of BSA (10mg/ml), 1 µl of RNAse (10mg/ml), 2 µl of spermidine (5mM) and a volume of sterile distilled water to make the final volume 50 µl. This mixture was incubated overnight at 37°C.

Before preparation of the gel for Southern blotting, a mini gel was prepared to analyse cut and uncut DNA. This was to check the quality and loadings of the DNA and verify that DNA had been digested, where applicable. To do this, the procedure described below was scaled down and after gel electrophoresis, the gel was stained with ethidium bromide for 30 minutes, destained with water and visualised using a short wavelength trans-illuminator.

For Southern blot analysis, a horizontal 1% (w/v) agarose gel in 1 x TAE buffer (0.4m Tris pH 8.2, 0.2m Sodium acetate, 10mM EDTA) was prepared (dimensions 20cm x 25.0cm x 0.5 cm). Prior to electrophoresis, 2 µl of loading dye was added to digested DNA samples.

Human liver DNA was loaded alongside human cell line DNA to act as a control. Using the BRL horizontal electrophoresis model 41, the DNA samples were electrophoresed

at 45V for 15-20 hours or until the dye front was 5cm from the edge of the gel. The gel equipment was disassembled and the gel denatured in denaturing solution (0.5M NaOH, 1.5M NaCl) and neutralised in neutralising solution (2M NaCl, 1m tris HCl, pH 5.5) for 45 minutes and 1 hour respectively.

The DNA was transferred to hybond paper by capilliary action in 10 x SSC (1.5 M NaCl, 0.15M tri-sodium citrate) as described by Southern (1975). The filter was then baked at 80°C for 2 hours in a vacuum oven to fix the DNA. The gel was checked after this proces in order to confirm the successful transfer of the DNA. To do this the gel was stained in ethidium bromide for 1 hour, washed with water and viewed using a short wavelength transilluminator.

The hybond filter was prehybridised at 65°C for 4-6 hours in pre-hybridisation mix in a heat-sealed plastic bag. A small incision was made in one of the top corners of the bag containing the filter and the prehybridisation mixture was removed. Hybridisation mixture at 65°C was then added, taking care not to trap any bubbles. The bag was sealed once again and incubated for a further hour at 65°C. The probe, which had been radiolabelled as described in Section 2.6.7 was denatured by heating to 100°C for 5 minutes and added to the bag. The bag was then sealed and the filter and probe allowed to hybridise for 16-24 hours at 65°C.

Following this, the filter was washed for 15 minutes in 2 x SSC (0.3M NaCl, 0.03M tri-sodium citrate), 0.1% (w/v) SDS and 0.1% (w/v) NaPPi, followed by a further 3 x 20 minute washes to remove the unbound probe. The filters were rinsed in 2 x SSC (0.3M NaCl, 0.03M tri-sodium citrate), air dried, wrapped in saranwrap and autoradiographed using Kodak Xar5 film.

Pre-Hybridisation Mix

5 x SSC
4 x Denhardts
10% (w/v) dextran sulphate
0.1% (w/v) SDS
0.1% (w/v)NaPPi
50 µg herring sperm DNA

Hybridisation Mix

5 x SSC
4 x Denhardts solution
10% (w/v) dextran sulphate
0.1% SDS
0.1% Na PPi

Loading Dye

0.4% bromophenol blue (w/v)
0.4% xylene cyamol (w/v)
50% glycerol (v/v)
1mM EDTA

Denhardts Solution

Ficoll 10mg/ml
PVP 10mg/ml
BSA 10mg/ml

2.6.5 Northern Blot Analysis

For northern blot analysis, a horizontal 1.5% agarose gel in MOPS buffer (0.2M MOPS, 50µM NaOAc, 10mM EDTA, pH 7.0) containing 18% formaldehyde solution from a stock formaldehyde solution of 40% (v/v) was prepared. RNA samples were prepared by adding 3 volumes of sample buffer to the volume of RNA required to give a final concentration of 15 µg of RNA and heating to 50°C for 30 minutes.

The loading dye was added at a one-tenth volume prior to loading. The RNA samples were electrophoresed at 45V for 15-20 hours or until the dye front was 5cm from the edge of the gel, in MOPS buffer, using a BRL horizontal electrophoresis model 41 (BRL).

The RNA was transferred by capilliary action in 10 x SSC (1.5M NaCl, 0.15 tri-sodium citrate) to hybond paper as described by Southern (1975). The hybond filter was rinsed in 2 x SSC (0.3M NaCl, 0.03 M tri-sodium citrate) and allowed to air dry before

irradiating it with U.V. from a short wavelength transilluminator for 2.5 minutes. The filter was then baked for an hour at 80°C.

In order to probe the filter, it was incubated for 4-6 hours in a sealed bag with pre-hybridisation mix at 68°C. The prehybridisation mixture was removed and hybridisation mixture added. The filter was incubated for a further hour at 68°C before adding the probe which was denatured just before adding to the filter, by heating to 100°C for 5 minutes. The hybridisation was allowed to proceed overnight. Following this, the filter was washed for 15 minutes in 2 x SSC (0.3M NaCl, 0.03M tri-sodium citrate) and for 2 x 30 minute periods in 2 x SSC (0.3M NaCl, 0.03M tri-sodium citrate) containing 0.1% (w/v) SDS and 0.1% (w/v) NaPPi. The filter was air dried, wrapped in saranwrap and autoradiographed using a Kodak Xar5 film.

Prehybridisation Mix

6 x SSC
2 x Denhardts
0.1% (w/v) SDS
0.1% Na PPi
50 µg herring sperm DNA

Hybridisation Mix

6 x SSC
2 x Denhardts
0.1% (w/v) dextran sulphate
0.1% (w/v) SDS
0.1% (w/v) Na PPi

Sample buffer

0.1M MOPS
25 mM NaOAc
5mM EDTA
pH 7.0

Loading dye

0.4% bromophenol blue (w/v)
0.4% xylene cyanol (w/v)
50 % glycerol (v/v)
1 mM EDTA

2.6.6 RNA slot blot analysis

RNA was extracted by lysis in guanidine thiocyanate and centrifugation in a caesium chloride gradient(Maniatis et al, 1982). Duplicate slot blots were made with five doubling dilutions of total RNA (10µg to 0.625µg) onto Hybond. The filters were prepared and probed with a full length GST pi cDNA (Kano et al, 1987) as described in sec tion 2.6.5. Quntification of RNA was carried out by hybridisation with a human B (PHA 4.1) probe (Khalili et al, 1983). The blots were finally washed with 2x SSC (0.3M NaCl; 0.03M ti-sodium citrate) containing 0.1% (w/v) SDS at 65°C Kodak Xar film exposed at -70°C for 1-5 days was used for autoradiography. Relative levels of GST pi expression was determined by densitometry.

2.6.7 Radiolabelling of cDNA probes

Purified cDNA inserts contained within low melting point agarose were "oligo-labelled" according to the technique of Feinberg and Vogelstein (1983). Labelling was performed overnight at room temperature and 90 ng of DNA in a total volume of 30 µl was used. The necessary solutions and constituents of the labelling reaction are as follows:

Radiolabelling reaction mixture

OLB 3µl
BSA (1mg/ml) 2µl
Klenow enzyme 1 unit
^{32}PdCTP 50µCi

The procedure for labelling the probe is outlined below. 90ng of the cDNA to be labelled, contained in low melting point agarose, was heated to 100°C for 3 minutes. As the DNA was cooling 3 µl of OLB was added. The mixture was cooled to room temperature, whereupon 2 µl of BSA, 19 µl of dH_2O and 50 µCi ^{32}P dCTP were added. Finally the Klenow was added and the reaction left to proceed at room temperature overnight.

The amount of radioactivity incorporated into DNA was estimated by DEAE-cellulose paper chromatography in 0.3 M ammonium formate, pH 8.0. To do this, 1 µl of the labelling reaction was applied to a strip of DEAE cellulose and the chromatograph was run for approximately 30 minutes before air drying and autoradiographing for 30 minutes using Kodak Xar5 film. Probes that incorporated 50-100% of the radioactive nucleotides were used for northern and Southern blotting.

This was assessed by analysing the autoradiograph. Labelled probe remained at the origin while unincorporated nucleotides ran close to the solvent front.

OLB

OLB is a complex buffer consisting of solutions A, B and C which are mixed in the ratio 2:5:1 respectively. This stock solutions A, B and C can be stored for up to 3 months at -20°C.

Solution A

625ml of 2M tris HCl, pH 8.0 + 25 ml of 5m $MgCl_2$ + 350 ml of dH_2O + 18 ml of mercaptoethanol + 5 ml of dATP, dGTP and dTTP (each tri-phosphate is dissolved in 3mM Tris/HCl, 0.2mM EDTA [pH 7.0] to give a concentration of 0.1M).

Solution B

2M HEPES titrated to pH 6.6 with NaOH. This solution is stored at 4°C.

Solution C

Hexadeoxyribonucleotides suspended in 3mM Tris/HCl, 0.2mM EDTA (pH 7.0) to give a concentration of 90 O.D. units/ml. This is stored at -20°C.

2.7.0 Immunohistochemistry on fixed cell lines

The tumour cell lines under investigation were cultured on 6cm diameter tissue culture petri-dishes (Nunc). Cells were plated at a density of 10^6 cells and incubated for 36 hours to allow cells to adhere and be in the log stage of growth when they were fixed.

Cells were fixed with a 50:50 (v/v) solution of methanol and acetone by first washing the cells three times with cold PBS and then pouring 2ml of cold fixing solution onto the cells. The fixing solution was left on the cells for 2 minutes after which time it was

poured away. The plates were allowed to air dry before freezing them at -40°C.

When required, the fixed cells were thawed in such a way as to avoid fracturing of cells. To do this, 2 mls of fixing solution, that had been cooled on dry ice for 30 minutes, was poured onto the cells as soon as they were taken out of the freezer. This was left on the cells for 10 minutes before pouring away and allowing the plates of fixed cells to air dry.

Sera containing antibody against the antigen under investigation was serially diluted in PBS containing 10% FCS. A grid was marked on the underside of the plate and 1 µl of each antibody dilution was spotted onto a square on the grid. This was incubated in a humid atmosphere for 2 hours at room temperature before washing the plates five times with PBS.

Following this, the plates were incubated for 1 hour in a humid environment at room temperature with a 1:200 (v/v) solution of antirabbit IgG antibody conjugated to horseradish peroxidase (Scottish Antibody Product Unit, Glasgow, Scotland) diluted with PBS containing 10% FCS (w/v). (If antibodies had been raised in mouse then the cells on the plates were incubated with antimouse IgG antibody conjugated to horseradish peroxidase [Scottish Antibody Product Unit, Glasgow, Scotland]).

Plates were again washed 5 times with PBS before adding the substrate diaminobenzene at a concentration of 1mg/ml in PBS to which hydrogen peroxide, at a 1:5000 (v/v) dilution was added immediately before developing the plates.

This was left for 20 minutes to allow the colour to develop. The substrate was washed off with distilled water and the stained cells viewed and photographed. The controls used in this type of analysis were pre-immune serum from the animal in which the antibodies under investigations had been raised.

2.8.0 GST activity determination

Enzyme assays were either performed automatically using a Cobas Fara centrifugal analyser or were determined by a manual method on a Schimadzu MPS 200 spectrophotometer. The method used was essentially the same as that described by Habig et al (1984b) where 1,chloro-2,4-dinitrobenzene was used as a substrate and using the Cobas Fara, the assays were normally performed in triplicate.

Assays were performed in 100mM sodium phosphate buffer pH 6.5. Samples were pre-incubated with GSH prior to initiation of the reaction by addition of CDNB. Final concentrations of GSH and CDNB were 2mM and 1mM respectively.

The conjugation of CDNB with GSH was measured by recording the absorbance of the reaction mixture at 340nm. A series of absorbance readings were taken. The first was taken 10 seconds after the reaction had been started and the other 7 at 5 second intervals thereafter. The enzyme activities measured were given as µmoles of product formed/min/mg protein.

The non-enzymatic conjugation of CDNB with GSH was subtracted from the enzymatic conjugation. Using the Cobas Fara, linear regression analysis was performed using the manufacturers kinetic rate programme for the GST assay. A preprogrammed conversion factor was used and data from the CDNB assay was presented as change in absorbance (ΔA)/min/ml. The extinction co-efficient of 38 allowed conversion of these units to µmoles/min/mg protein.

Using the manual method, the reaction was carried out in 100mM sodium phosphate buffer, pH 6.5 and the final concentrations of GSH and CDNB were 2mM and 1mM respectively. The non-enzymatic conjugation of CDNB to GSH was recorded at 340nm before the sample was added to the test cuvette. The enzymatic conjugation of CDNB to GSH was then recorded at 340nm. The change in absorbance (ΔA) was calculated per minute and converted to µmoles using the extinction coefficient of 38. The units of enzyme activity were given as µmoles of product formed/min/mg protein.

2.9.0 Reverse phase HPLC and subsequent Western blot analysis of cell line cytosols

The levels of B_1 and B_2 subunits were investigated using reverse hplc analysis according to the method of Ostlund Farrants et al (1987) followed by western blot analysis according to the method described in Section 2.5.0. A Waters reverse phase HPLC (Model 510) machine was used. The column (Waters µ-Bondapak C-18 reverse phase column 0.3cm x30cm) was washed through with 70% acetonitrile .

Two solvent mixtures (A and B) were then attached to the equipment. (A)

consisted of 200 mls of acetonitrile, 300 mls of distilled water and 0.5 ml of trifluoroacetate. (B) consisted of 350mls of acetonitrile, 150 mls of distilled water and 0.5 ml of trifluoroacetate. Both solutions were filtered prior to use.

The equipment was then programmed to give the required mix of solutions A and B in order to create a gradient which washed through the column. Before switching in the gradient, a mixture of purified B_1 and B_2 subunits was applied to the column. The preset fraction collector and pen recorder were then switched on at the same time as the solvent flow which created the gradient.

The absorbance of the protein eluted from the column, was measured at 280 nm. After analysis of the purified B_1 and B_2 subunits, the column was washed through for 2 hours with 70% acetonitrile. Cell cytosol containing 500 µg of protein was then loaded onto the column. Trifluoracetate diluted 1:10 (v/v) was added to the cytosol before addition to the column, at a ratio of 1:3 (v/v) trifluoracetate to cytosol. The cytosol was eluted from the column as described for the B_1 and B_2 subunits.

Cell cytosol protein samples eluting in the same range of fraction numbers as B_1 and B_2 subunits were collected. The above procedure was repeated for the cytosol from several cell lines. Purified B_1 and B_2 subunits were always used to calibrate the system prior to adding sample cytosol.

The samples were prepared for western blot analysis as follows: The isolated fractions were freeze dried before adding 40 µl of a 1:10 (v/v) dilution of buffer (1.5 m Tris/HCl, 0.5% (w/v) SDS, pH 8.8) and 20 µl of boiling mix (10% [v/v] stacking gel buffer, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 10% (v/v) glycerol and 0.005% (w/v) bromophenol blue).

SDS polyacrylamide gel electrophoresis (Section 2.4.0) and western blot analysis (Section 2.5.0) were then carried out and the blots probed with an anti human B_1 antibodies.

LIST OF SUPPLIERS

The chemicals and equipment used were obtained from the following sources:

Amersham Internatonal,Amersham, Buckinghamshire, U.K.

$P^{32}CTP$; dATP; dGTP; dTTP; Hexadeoxyribonucleotides; ^{125}I protein A; Hybond

Anderman & Co Ltd., Kingston-Upon-Thames, Surrey.

Nitrocellulose.

Becton-Dickson, Cowly, Oxfordshire, U.K.

Sterile syringes.

B.D.H. Chemicals Ltd., Thonliebanh, Glasgow.

EDTA; Hydrogen peroxide; Chloroform; Ammonium acetate; Ammonium formate; Acetone; Methanol; NaCl; KCl; NaH_2PO_3; KPO_4LiCl; $MgCl_2$; $NaCO_3NaOH$; $CuSO_4NaK$ tartrate; LiCl; Sodium citrate; Sodium tri-citrate; Sodium acetate;

Biorad Laboratories, Watford, Hertfordshire.

Ammonium persulphate; 4-chloro-1-napthol; Tween 20.

B.R.L., Uxbridge, Middlesex, U.K.

EcoR1; BamH1; Hind111; Pst1; Costar serile 96-well tissue culture plates; Formamide; Phenol (redistilled nucleic acid grade); Urea; Klenow; RNase; Pronase; Spermidine; Herring sperm DNA; Denhardts.

Fisons, Loughborough, U.K.

Mercaptoethanol.

GIBCO, Ltd., Paisley, Scotland.

Foetal calf serum; RPMI 1640 media; Nunc sterile tissue culture flasks and plates Steptomycin; Penicillin; New born calf serum; Sterile Falcon tubes; Trypsin.

Koch Light Laboratories, Colinbrook, Berkshire, U.K.

Cumene hydroperoxide.

Kodak Ltd., Kirby, Liverpool, U.K.

Kodak Xar film.

May and Baker, Dagenam, Kent, U.K.

Absolute alcohol; Acetic Acid.

Millipore (U.K.) Ltd., Harrow, Middlesex, U.K.

Ultrafiltration membranes (0.45μM) ; Sterile syringe filters (0.22 μM)

Oxoid, Basingstoke, Hampshire, U.K.

Phosphate buffered saline tablets.

Pharmacia,Milton Keynes,U.K.

Ficoll; Dextran sulphate

Raeburn Chemicals Ltd., Walkerburn, Peebleshire, Scotland.

Acetonitrile.

Scientific Furnishings Ltd.,Macclesfield, Chesire, U.K.

Hemocytometers.

Sigma Chemical Co., Poole, Dorset, U.K.

Insulin; Dimethyl sulphoxide; CDNB; Ethacrynic acid; nigrosin; MTT; BSA; Acrylamide; Bisacrylamide; Ethidium bromide; GSH; Folin Ciocalteau; DTT; Tris-

HCl; SDS; TEMED; Glycine; Agarose; Low melting point agarose; NaPPi; Xylene cyanol; HEPES; PVP; DEPC; MOPS; Formaldehyde; Glycerol; Coomasie brilliant blue R; CDTA; Glycerol; Diaminobenzene.

CHAPTER 3

THE ROLE OF THE GLUTATHIONE S-TRANSFERASES IN RESISTANCE TO CYTOTOXIC COMPOUNDS

3.1.0 INTRODUCTION

Intrinsic and acquired drug resistance are of central importance in clinical oncology. The term intrinsic, or *de novo*, resistance is used to describe the situation where a cellular population which has not been exposed previously to any chemotherapeutic agent, can tolerate a particular drug at concentrations that are toxic to other cellular populations. In such cases the resistant population is thought to possess certain inherent "features" that allows it to survive drug concentrations that are cytotoxic to other cells. It is thought that the causal factors of intrinsic drug resistance, in a large number of cases, have evolved as a result of selection pressures that are totally unrelated to the drug to which tolerance is observed. Unfortunately, the cellular mechanisms and factors that bestow this resistance are, to a large degree, unknown.

By contrast to intrinsic drug resistance, acquired resistance describes the process whereby a resistant cell population emerges from one which had initially been sensitive to the drug in question and is a direct result of exposure to the particular compound. In cancer chemotherapy, the major problem with acquired drug resistance is that as well as resistance to the compound to which the cellular population was initially exposed, resistance to a range of structurally and functionally unrelated drugs is often encountered.

Factors that may account for this clinical observation have become apparent in the last few years and it has been found that they are often absent from a cellular population or present at low levels, before the cells are challenged with the drug. It has also emerged that several mechanisms may operate simultaneously and which mechanism is responsible for resistance is likely to depend on the nature of the drug to which cells were initially exposed.

In recent years there have been a growing number of reports that the glutathione S-transferases (GSTs) are involved in the process of drug resistance. However, in the majority of cases it has not been ascertained whether the reported overexpression of GSTs is a cause or an effect of drug resistance. In addition, only two of the antitumour agents commonly associated with the drug resistance have actually been shown to be substrates for the GST isoenzymes. One further criticism that can be made of the work in this area, is that although there are a growing number of reports that there is an increase in overall GST activity in several drug resistant cell lines, the identity of the GST subunits that are overexpressed is seldom investigated.

In an attempt to clarify the role of the GSTs in both intrinsic and acquired drug resistance several tumour cell line models have been analysed in the work presented below.

3.2.0 RESULTS AND DISCUSSION

3.2.1 Intrinsic Resistance of Human Tumour Cell Lines to Alkylating Agents.

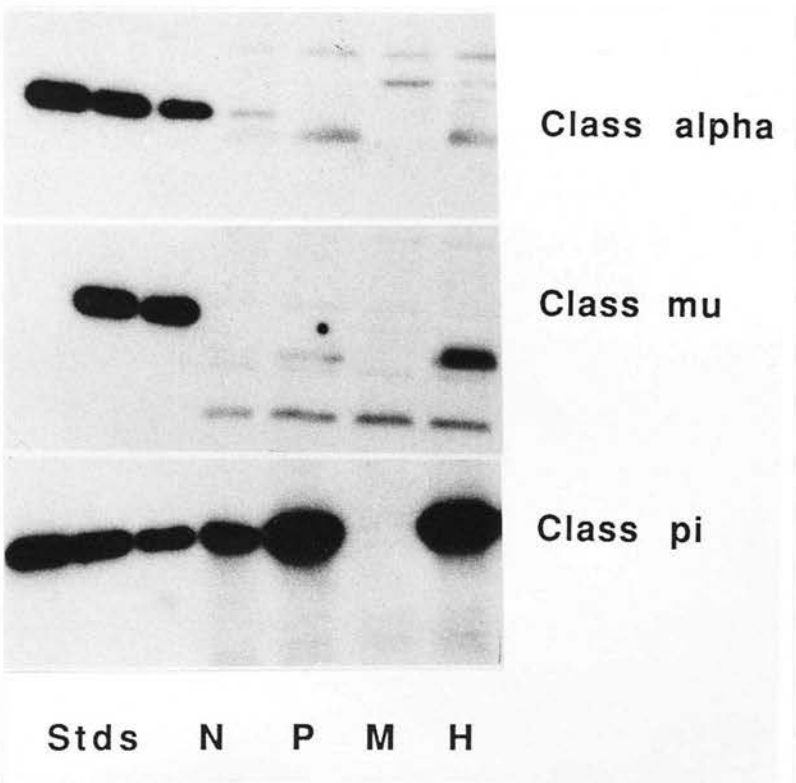
In order to study the potential role the GST may play in clinically encountered intrinsic drug resistance, cytosolic fractions from four human tumour cell lines were prepared according to the Materials and Methods (Section 2.2.1). These cell lines were PE04, HT29, MCF7 and NCIH322 which had originally been derived from human ovarian colonic, breast and lung tumours respectively. The cytosolic fractions from these cells were subjected to western blot analysis according to the Materials and Methods (Section 2.5.0), and the blots were probed with antibodies that had been raised to purified human GST from the three cytosolic classes. The results are shown in Figure 3.01.

From this analysis, it is evident that of the four cell lines, MCF7 expresses the lowest levels of GST. In this cell line there is no detectable class alpha, mu or pi expression. These results are comparable to those obtained by other investigators working on this cell line (Batist et al 1986). These workers estimated the low overall GST activity of this cell line to be 3.6 nmol/min/mg.

Figure 3.01 GST subunit expression in the cytosol of four human tumour cell lines.

Cells were harvested from confluent tissue culture flasks as described in Section 2.1.5 and prepared as described in Section 2.2.1

25μg of soluble protein was subjected to SDS/PAGE, transferred to nitrocellulose and probed with specific antibodies raised against purified subunits from the three known human cytosolic GST classes: alpha (B_1); mu (μ) and pi (π); Stds = Standard (purified protein); N = NCI H322; P = PE04; M = MCF-7 and H = HT29.



The other three cell lines, HT29, NCIH322 and PEO4 all express relatively high levels of the class pi enzyme. Of the three human cytosolic GST classes, the class pi subunit has been shown to have the widest distribution in different tumour cell lines (Batist et al, 1986; Deffie et al, 1988; Gupta et al, 1989). The significance of this is unknown, although it has been suggested that in tumour tissue, surgically removed from patients, the class pi subunit is elevated compared to normal surrounding tissue (Soma et al , 1986; Di Ilio et al, 1987; Moscow et al, 1989; Howie et al, 1990). Since most tumour cell lines are established from surgically-removed malignant tissue, it may not be surprising that overexpression of the pi subunit is commonly encountered in tumour cell lines. It is also interesting that high levels of the class pi subunit are observed in tumour types usually classed as being intrinsically resistant to chemotherapeutic agents, for example, colonic tumour tissue (Kodate et al, 1986; Moscow et al, 1989).

Relatively little class alpha or mu subunit expression is detectable in any of the four cell lines analysed, although there is a low level of expression of the class alpha subunit(s) in the NCIH322 cell line (Figure 3.01).

The four cell lines analysed in Figure 3.01 were studied with respect to their sensitivity to several toxic compounds. The MTT assay was used to test the sensitivity of PEO4, MCF7, HT29 and NCIH322 to 1-chloro-2,4-dinitrobenzene, ethacrynic acid and chlorambucil, TheMTT assay uses the fact that the dehydrogenase enzymes in the mitochondria of live but not dead cells can metabolise the compound [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], (MTT) to its tetrazolium salt. The latter product is blue and is used to assess the percentage of live cells by means of colorimetry (Carmichael,et al, 1987). Only HT29 and MCF7 were compared graphically to avoid confusion (Figs 3.02 to 3.04). These two cell lines were chosen since they exemplified cell lines with high and low GST content respectively. As can be seen, MCF7 is more sensitive to the effects of CDNB, CHP and chlorambucil compared to HT29 as judged by the LD_{50} for these compounds; the LD_{50} is a measure of the dose of drug which is lethal to 50% of the cells.

Although this evidence is very circumstantial, it does perhaps suggest that the intrinsic drug detoxification capacity, as exemplified in this work by GSTs, may determine to some extent, the intrinsic resistance of a tumour population. It may be that the drug

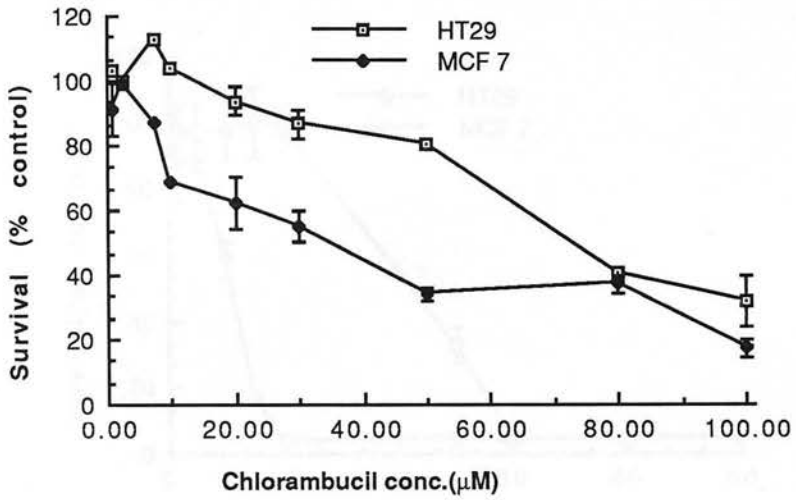


Fig.3.02 Sensitivity of HT29 and MCF7 human tumour lines to chlorambucil.
MTT assays were carried out as described in the Materials and Methods. The values shown are the mean of triplcate determinations.

$L.D._{50}$ MCF7 = 38μM chlorambucil

$L.D._{50}$ HT29 = 75μM chlorambucil

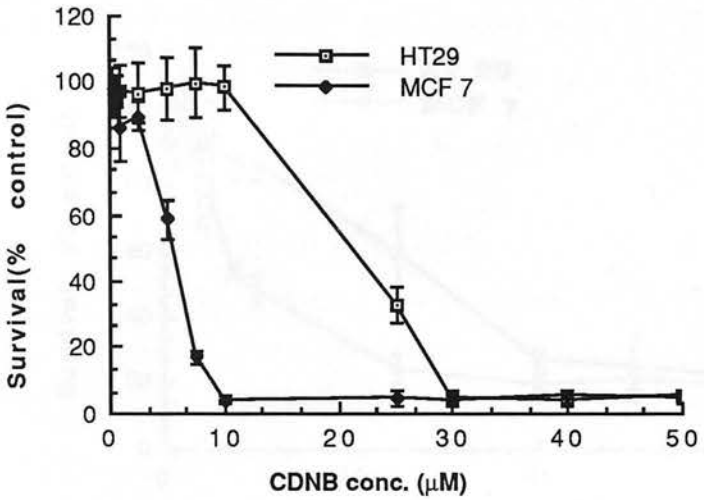


Fig. 3.03 Sensitivities of HT29 and MCF7 human tumour cell lines to CDNB.
MTT assays were carried out as described in the Materials and Methods. The values shown are the mean of triplicate determinations.

$L.D._{50}$ MCF7 = 6.0μM CDNB

$L.D._{50}$ HT29 = 21.5μM CDNB

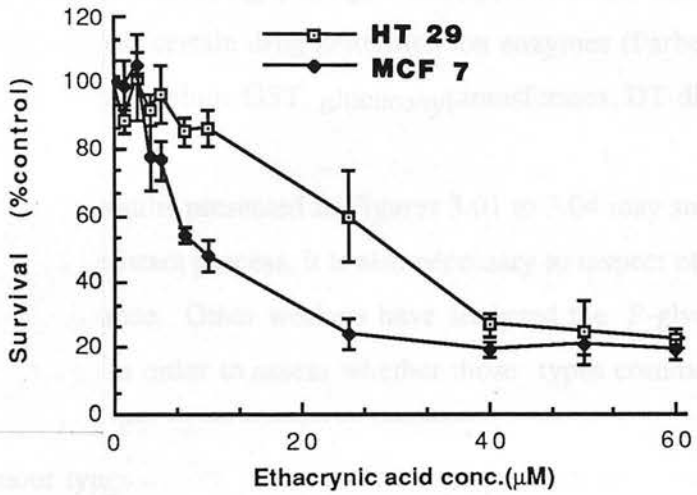


Fig.3.04 **Sensitivities of HT29 and MCF7 human tumour cell lines to ethacrynic acid.**
MTT assays were carried out as described in the Materials and Methods. The values shown are the mean of triplicate determinations.

$L.D._{50}$ MCF7 = 10μM ethacrynic acid

$L.D._{50}$ HT29 = 30μM ethacrynic acid

detoxification capacity of a cell changes as a result of the carcinogenesis process.

It has already been shown that preneoplastic foci in rat liver, which can be induced by various carcinogens including 2-acetylaminofluorene and 3-methyl-4-dimethylamine azobenzene, overexpress certain drug detoxification enzymes (Farber, 1984;Cowan et al, 1986;Carr, 1987). These include GST, glucuronyl-transferases, DT-diaphorase and epoxide hydrolase.

Although the results presented in Figures 3.01 to 3.04 may suggest a role for GSTs in the intrinsic drug resistant process, it is also necessary to inspect other factors thought to be involved in resistance. Other workers have analysed the P-glycoprotein contents of various tumour types in order to assess whether those types commonly classed as being intrinsically resistant, and so refractory to treatment, express higher levels of this protein than other tumour types usually classed as being responsive to treatment (Goldstein et al, 1989).

The picture to emerge from analyses of this nature is far from clear cut. However, in general it appears that tumour types that are particularly refractory to treatment express higher levels of P-glycoprotein than their more treatable counterparts. For example, colonic tumour tissue has been investigated by several workers (Moscow et al, 1989; Goldstein et al, 1989) and the majority of tumours analysed were seen to express high levels of mdr 1 mRNA. However, the data presented by these workers also showed that some intrinsically resistant colonic tumours seem to express very low levels of P-glycoprotein which leads to speculation that other resistance mechanisms are operational in these cases.

GSTs have also been analysed with respect to their expression in different tumour types. Again, the results are not at all clear cut. There does however seem to be a pattern emerging that the more intrinsically resistant tumours such as lung and colon express higher GST levels (Kodate et al, 1986; Moscow et al, 1989).

3.2.2 Acquired Resistance of Human Tumour Cell Lines

From the work on the four different cell-lines that has been detailed above, MCF7 and NCIH322 were chosen as parent cell lines from which drug resistant variants would be

derived in order to study acquired drug resistance. Since much of the work described in the literature has failed to show that the drugs used to derive resistant cell lines can actually act as GST substrates, in this work, known GST substrates were used to derive resistant cell lines.

(i) Selection of an MCF7 Subline Resistant to Ethacrynic Acid

The MCF7 cell line has been used by various workers to create variant sublines resistant to several compounds (Batist et al, 1986; Wheelan et al, 1990). From work of this nature, the MCF7 cell line appeared to be a reasonable choice because drug resistant lines have been successfully achieved in the past. The MCF7 cell line also seemed a very suitable model in which to study GSTs since the parent line had been shown to possess low levels of these enzymes. It had also been shown that the class pi enzyme at least, could be induced in this cell line (Batist et al, 1986).

The derivation of an ethacrynic acid resistant MCF7 variant is detailed in Figure 3.05. Unfortunately after this derivation had been in process for four months, the ethacrynic acid-treated cell line became contaminated with a yeast infection. The progress with this cell line had been so slow that only limited frozen stocks had been obtained. However, a minimum number of cells had been harvested which enabled a cytosolic fraction to be prepared.

A western blot was carried out on the cell line and the results are shown in Figure 3.06. As can be seen, there has been an elevation of the human mu subunit. This result is perhaps surprising since ethacrynic acid is a substrate for certain class alpha and pi enzymes. It is difficult to offer an explanation for this result due to lack of other available information. There are at present no published results showing that mu can be elevated in MCF7 in response to any of the chemicals that have been used to derive drug resistant variants.

Recently, however, there has been a report that a class mu subunit is elevated in a rat gliosarcoma cell line made resistant to BCNU (Smith et al, 1989). In rat preneoplastic foci it has also been noted that rat class mu proteins can be elevated and it has been postulated that elevation is observed as a result of challenge by carcinogenic compounds (Cowan 1986; Carr 1987; Beer & Pitot 1987).

Although ethacrynic acid is not a substrate for the mu class enzymes, it is possible

FIGURE 3.05 Derivation of human tumour cell lines resistant to cytotoxic GST substrates

a = MCF 7 cell line resistant to ethacrynic acid.

b = NCI H322 cell line resistant to CDNB,

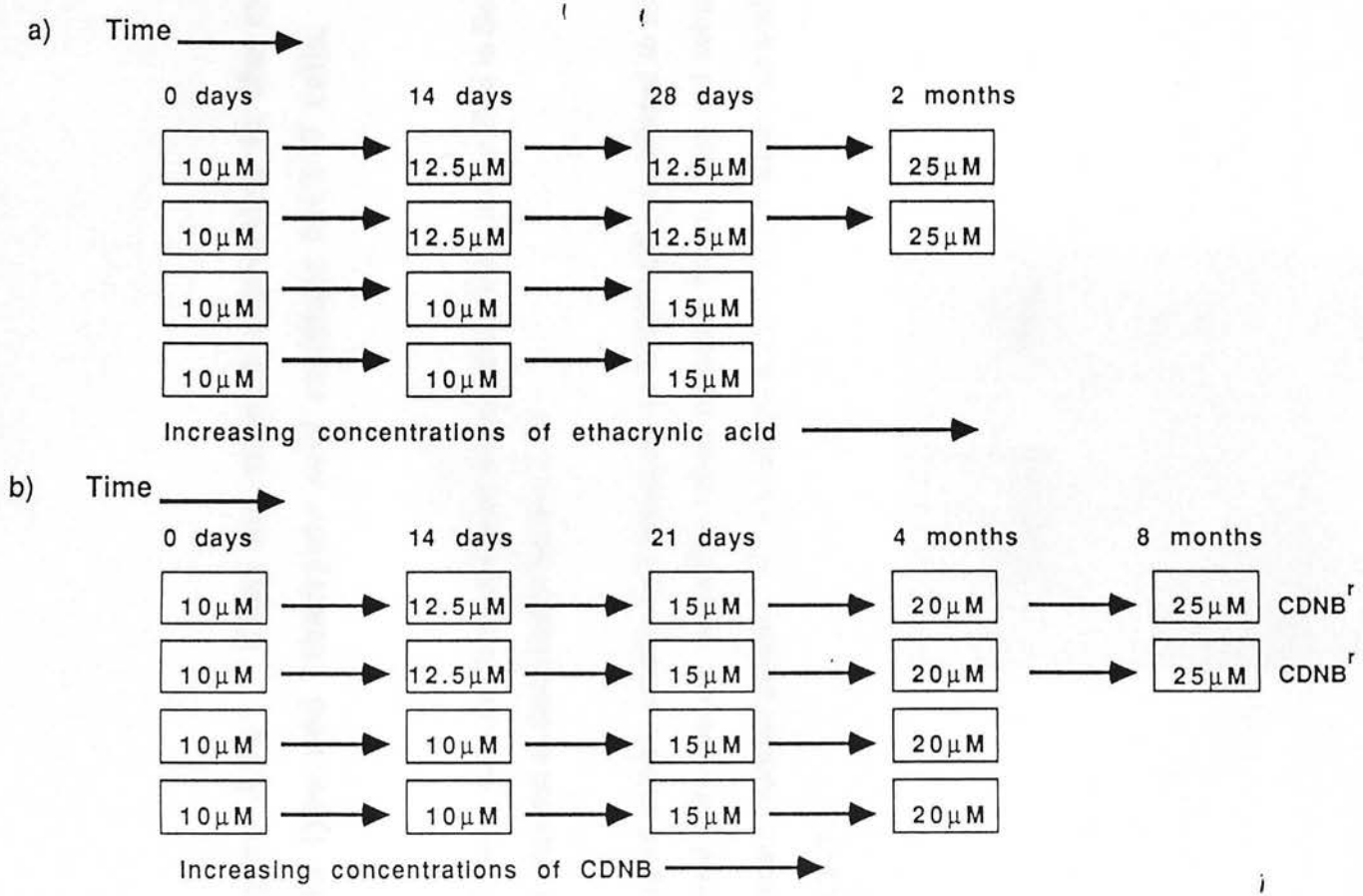
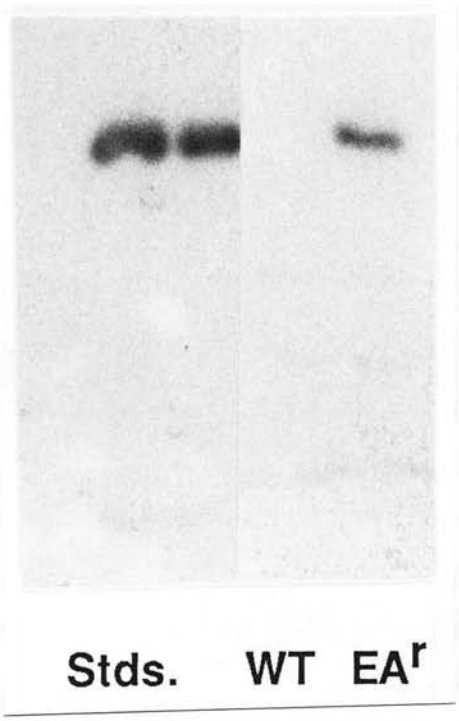


Figure 3.06 Class mu subunit expression in the cytosols of wild type and ethacrynic acid resistant MCF-7 cells.

Cells were harvested from confluent tissue culture flasks as described in Section 2.1.5 and prepared as described in Section 2.2.1

25μg of soluble protein was subjected to SDS/PAGE, transferred to nitrocellulose and probed with specific antibodies raised against a human class mu subunit (μ); Stds = Standard (purified protein); WT = wild type MCF-7; EA^r = ethacrynic acid resistant MCF-7.



that in the model described above, induction of the mu class subunit has occurred in order to fulfil a non-enzymic function. It has previously been shown (Yamada & Kaplowitz 1980) that hepatic class mu enzymes can bind ethacrynic acid covalently. In the model generated during this work, resistance to ethacrynic acid may have occurred as a result of increased drug binding resulting from the overexpressed class mu subunit.

It is not possible to state the fold resistance exhibited by the MCF7 variant cell line to ethacrynic acid, since it was not possible to carry out an MTT assay on this cell line. The only available information on resistance is that 100% of the wild type cells were killed when subjected to the same concentrations of ethacrynic acid that the variant cell line could tolerate (ie. 25μm ethacrynic acid).

(ii) Selection of an NCI H322 Cell Line Resistant to CDNB

As previously mentioned the NCI H322 cell line was also chosen as a parent line from which to derive a variant,resistant to known GST substrates. NCI H322 is a human lung tumour cell line derived from an adenocarcinoma of the lung and is very similar to Clara cells in the features it possesses (Carmichael et al, 1988). The GST concentration of this cell line is comparable to the concentrations observed in preparations of human lung (Mukhtar et al 1981).

This cell line was chosen for several reasons. Firstly, it was derived from a human tumour type which, as described in the Introduction is one that initially responds well to chemotherapy regimens but which often is associated with relapse as a consequence of emerging drug resistant populations (ie. acquired drug resistance). Secondly, the drug detoxification capacity of this cell line has already been examined since it has been used previously to study the cytotoxic effects of a number of compounds (Falzon et al 1986; Weibel et al, 1986). Thirdly, the parent cell line expresses class pi and, to a much lesser extent alpha class subunits (see Figure 3.01), showing that it possesses the transcriptional factors required for the expression of these two classes of enzyme.

CDNB, which is a general substrate for all three cytosolic GST classes was used to derive a resistant variant. The derivation process is outlined in Figure 3.05. Progress with this human lung tumour cell line proved to more successful than with the MCF7 line. After eight months of continual culture in the presence of an incrementally increasing

concentration of CDNB, a cell line was derived that exhibited 2-2.5 fold resistance to this compound compared to the parent line, as ascertained by the MTT assay (Figure 3.07).

Cells were frozen down at various stages of the derivation process to safeguard against contamination. At the 8 month stage of derivation, the CDNB resistant line, termed $CDNB^r$ was cultured in the absence of CDNB. The phenotype was found to be stable and the observed changes which are described below were also stable in the absence of CDNB for at least 3 months (see Figures 3.14 and 3.15).

The stability of the phenotype is discussed in more detail in Section 3.2.5. Since frozen stocks of the $CDNB^r$ cell line were available, analysis was only done on cells that had been in culture for no longer than 3 months. After this time, fresh cells were cultured from the frozen stocks.

Figure 3.08 shows the microscopic appearance of both the wild type and CDNB resistant ($CDNB^r$) cell lines. The cells of the resistant population display a more spiked morphology than the sensitive parental cells but the reason for this is unknown. However, there has been a report that cells treated with a tumour promoting compound show a change in their appearance similar to those reported in this thesis (Kitijama et al, 1988).

The underlying mechanisms responsible for these changes are unknown. It is also unknown whether these changes have any role in the resistance these cells exhibit to CDNB. It may be that the cytotoxic effects of the drug have caused alterations in membrane or cytoskeletal structure, which may in some way allow the cells to resist concentrations of CDNB that would normally be toxic.

3.2.3. Expression of the Human Cytosolic GST in the CDNB Resistant NCIH322 Cells.

Initially the overall GST activity was analysed according to the method of Habig et al, (1974b) using CDNB as a diagnostic substrate. A marked increase in activity towards CDNB was observed in the resistant subline (Figure 3.09). The parent cell line had an overall GST activity of 28$\pm$ 10 units and the $CDNB^r$ line had an overall GST activity of 375$\pm$30 units. Other workers have reported increases in GST activity in many drug-resistant cell lines (Batist et al , 1986; McGowan & Fox 1986; Deffie et al, 1988). Typically

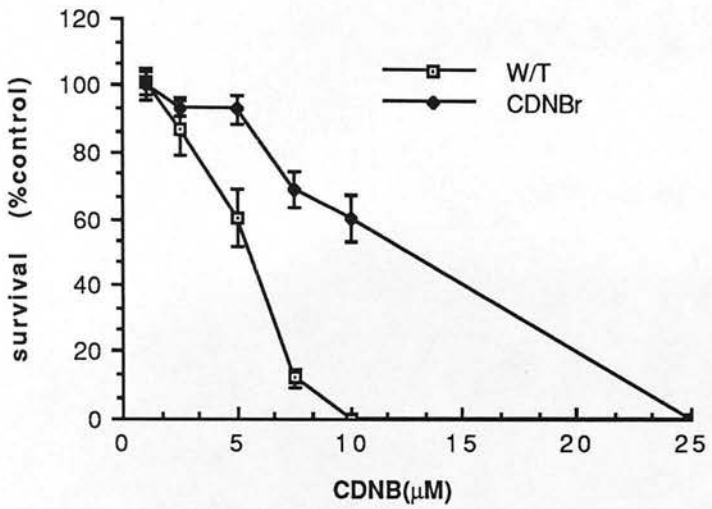


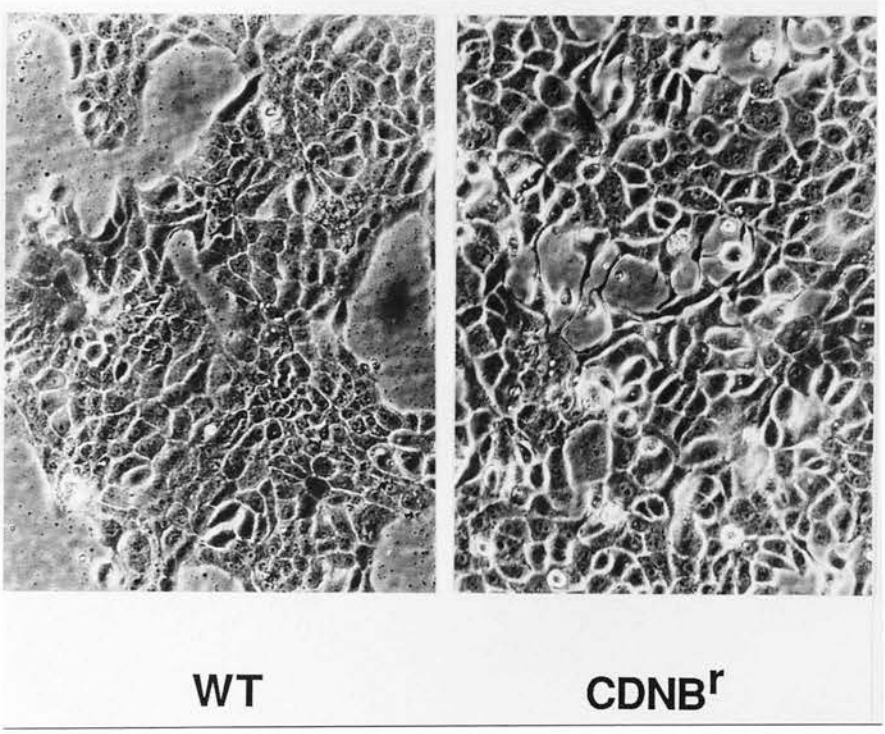
Fig.3.07 Sensitivity of wild type (W/T) and CDNB resistant (CDNBr) cell lines to CDNB.
MTT assays were carried out as described in the Materials and Methods. The values shown are the mean of triplicate determinations.

$L.D._{50}$ WT = 5.5μM CDNB.

$L.D._{50}$ CDNBr = 12.0μM CDNB.

Figure 3.08 Microscopic appearance of wild type and CDNB resistant NCI H322 cells.

Cells were viewed at a x 200 magnification and photographed.



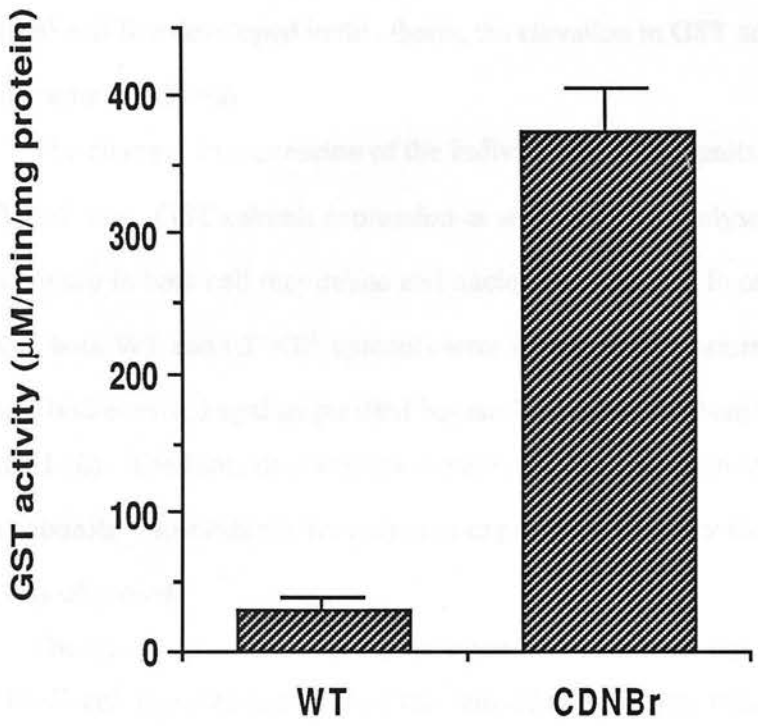


Fig 3.09. Overall Gst activity in WT and CDNB^r.

Cells were harvested and cell cytosol fractions prepared as described in the Materials andMethods (sections 2.1.5 and 2.2.1). GST activity was assessed using CDNB as a substrate according to the Materials and Methods (section 2.8.0)

increases in the GST activity which have been observed in these studies is between 1.5 and 5.0-fold, with the notable exception of the adriamycin resistant MCF 7 cell line (Batist et al, 1986) where GST activity was found to be elevated 15 to 45-fold. In the $CDNB^r$ cell line developed in this thesis, the elevation in GST activity towards CDNB is the order of 15-fold.

The changes in expression of the individual GST subunits were then investigated in the $CDNB^r$ line. GST subunit expression as well as being analysed in the cytosolic fraction was analysed in both cell membrane and nuclei preparations. In order to study the cytosolic fraction, both WT and $CDNB^r$ cytosols were subjected to western blot analysis and probed with antibodies raised against purified human GST subunits from the three cytosolic classes (Figure 3.10). The blots demonstrate a marked overexpression of both human class pi and alpha subunits. No evidence for class mu expression in either the wild type or $CDNB^r$ cell lines was observed.

The reason for absence of the mu class GST is unclear but it may be that the NCI H322 cell line was derived from an individual who was nulled for the mu subunit. It has already been shown that 45% of the human population are nulled for the mu subunit and it has been proposed that individuals with the null phenotype are more susceptible to lung cancer if they smoke (Seidegard et al, 1986).

Since CDNB is a general substrate for all classes of cytosolic GST (Table 1.4) , the overexpression of alpha and pi class subunits strongly indicates that, in this model, the overexpressed proteins are conferring resistance to CDNB. Although it is impossible to rule out other factors that may be conferring resistance, this information taken together with the results of Black et al (1990) are strong evidence in favour of this proposal. In the work by Black et al (1990) human cDNAs encoding π and B_1 subunits were cloned into vectors and when expressed in yeast cells were found to confer resistance to CDNB, cumene hydroperoxide, chlorambucil and adriamycin.

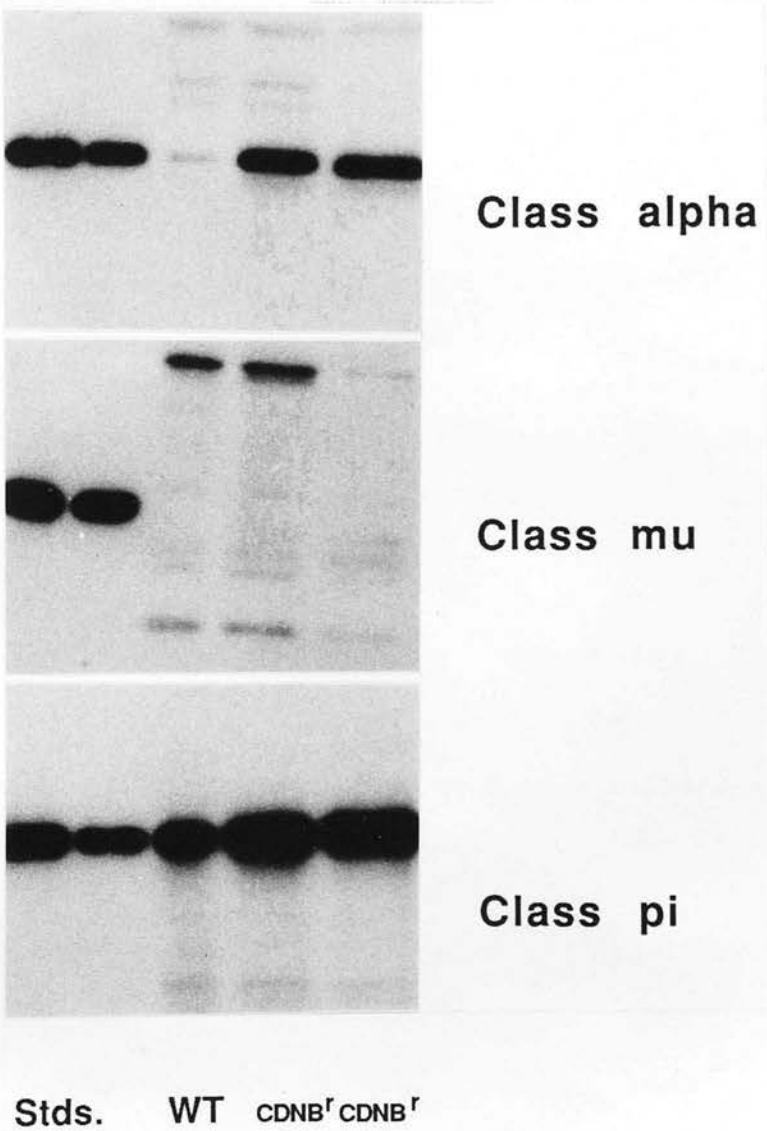
In the increasing number of drug resistant models reported in the literature where various GST subunits are overexpressed (see Table 1.5), it has been suggested that the GST may contribute to resistance by detoxifying drugs through catalysing the conjugation

Figure 3.10 GST subunit expression in the cytosol of wild type and CDNB resistant NCI H322 cells.

Cells were harvested from confluent tissue culture flasks as described in Section 2.1.5 and prepared as described Section 2.2.1

25μg of soluble protein was subjected to SDS/PAGE, transferred to nitrocellulose and probed with specific antibodies raised against subnits from the three known human cytosolic GST classes: alpha (B_1); mu (μ) and pi (π).

Stds = Standards (purified protein); WT = wild type NCI H322 cells; $CDNB^r$ = CDNB resistant NCI H322 cells.



of the drug with GSH (Hayes & Wolf, 1988). However, more supportive evidence for this would come from studies showing that the drugs in question are indeed GST substrates. Dulik et al, (1986) have isolated melphalan-glutathione conjugates whose formation was shown to be catalysed by certain GSTs, although the identities of the GST subunits responsible for catalysis were not established.

Smith et al (1989) have examined the role of GSTs in resistance to 1,3-bis(2-chloroethyl)-4-nitrosourea (BCNU). These workers derived a rat brain tumour line resistant to BCNU and when this cell line was analysed, elevated mu class GST activity was observed. Using a range of cytosolic GSTs they also showed that the rat class mu proteins were by far the best catalysts of BCNU denitrosation reactions, leading the authors to speculate that class mu overexpression was at least partially responsible for the observed resistance to BCNU.

Further and more comprehensive evidence that GSTs play a causal role in resistance to certain anticancer drugs would come from purifying and characterising the GSTs that are overexpressed in resistant cell lines. It would then be necessary to show that the GSTs in cell lines resistant to antitumour agents are capable of catalysing detoxification reactions involving the drug to which resistance is seen. Information of this nature is eagerly awaited.

3.2.4 Analysis of Alpha Class Subunit Expression in CDNB Resistant NCI H322 Cells

The western blot analysis presented in Figure 3.10 demonstrates a marked overexpression of both human class pi and alpha subunits. However, it is not possible to discriminate between the alpha class B_1 and B_2 GST subunits by western blotting. The data presented in Figure 3.10 do not indicate whether B_1 or B_2 or indeed both are elevated in the CDNBr cells.

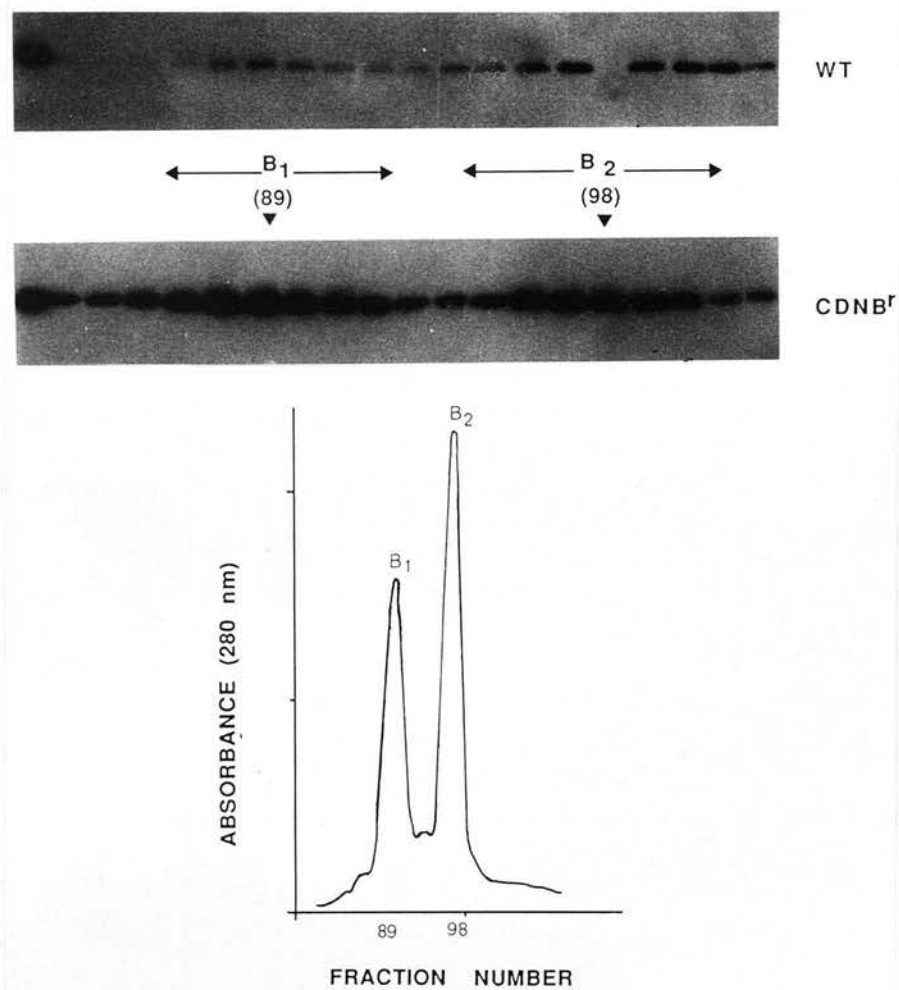
In order to clarify the situation with respect to B_1 and B_2 expression, whole cell cytosols of both WT and $CDNB^r$ cell lines were subjected to reverse phase HPLC analysis according to the method of Ostlund-Farrants et al (1987), since B_1 and B_2 alpha class subunits can be resolved by this technique (Hayes et al, 1990). The results are shown in Figure 3.11.

Purified human B_1 and B_2 subunits were initially applied to the μ-Bondapak HPLC

Figure 3.11 Purification of B_1 and B_2 subunits from wild type (WT) and CDNB resistant ($CDNB^r$) cell lines.

Cells were harvested from confluent tissue culture flasks as described in Section 2.1.5 and prepared as described in Section 2.2.1 The cytosolic fractions were subjected to HPLC analysis as described in Section 2.9.0. The purified protein fractions were subjected to SDS/PAGE, transferred to nitrocellulose and probed with specific antibodies to the human B_1 subunit.

89 and 98 = Fraction numbers giving highest absorbance reading at 280 nm during collection of fractions.



column to establish the retention times of those subunits. Immediately following calibration, whole cell cytosol from the WT cell line was applied to the column and subsequently eluted. The fraction numbers corresponding to those in which B_1 and B_2 had been eluted were collected. The column was washed, the procedure repeated for the standard B_1 and B_2 proteins, the column was washed again and the procedure was then repeated for whole cell $CDNB^r$ cytosol.

The protein concentration of WT and $CDNB^r$ cytosols loaded onto the column were equivalent. The fractions that had been collected for both WT and $CDNB^r$ lines were analysed by western blotting. As can be seen from Figure 3.11, both B_1 and B_2 are overexpressed in the resistant cells. The WT cell line contains slightly more B_2 subunit (approximately 2-fold) as judged by the intensity of the immunoreactive bands. However, in the $CDNB^r$ cell line, the reverse is true in that there seems to be slightly more B_1 than B_2 subunit overexpression

These data indicate that B_1 is elevated in the $CDNB^r$ line to a greater extent than B_2. If time had permitted, quantitative data regarding B_1 and B_2 overexpression could have been obtained using the R.I.A. technique developed by Hayes et al (1983); this technique also differentiates between B_1 and B_2 subunits.

The results shown in Figures 3.10 and 3.11 raise the question of which factors govern the regulation of the B_1 and B_2 subunits. It has already been shown that these two proteins, although 96% homologous, are the products of two different genes (Hayes et al, 1990). It may be that their regulation by certain regulatory factors in certain instances, is tightly linked and that factors leading to elevation in one subunit also lead to elevation in the other.

3.2.5. Stability of GST overexpression in the CDNB Resistant Cells.

The stability of overexpression of the B_1, B_2 and π subunits was investigated and correlated with the stability of the CDNB resistant phenotype. During the derivation of the $CDNB^r$ cell line, once resistance to CDNB had reached about 2-2.5-fold a further increase in resistance proved to be unattainable. At this stage CDNB was removed from the cells and

this cell line was termed CDNB^r. The analyses described above were carried out on these CDNB^r cells. Some cells however were maintained in the presence of CDNB.This cell line is termed CDNB^r(2) for convenience.

The GST profile of WT, CDNB^r and CDNB^r(2) was analysed one month after the CDNB had been removed from CDNB^r but maintained on CDNB^r(2). The results are shown in Figure 3.13. In the CDNB^r(2) cell line it was found that the expression of the class alpha GST had reverted back to the levels seen in the WT cell line despite the fact this cell line was grown in the presence of CDNB.

However in CDNB^r, after the initial eight month derivation process in the presence of CDNB, the class alpha protein expression remained elevated for at least three months in the absence of CDNB (Figs 3.14 and 3.15). The class pi subunit remained elevated in both CDNB^r and CDNB^r(2) irrespective of whether the cell line was grown in the presence or absence of the drug.

In terms of the stability of overexpression of the GST subunits, it would seem that π overexpression is stable whether or not CDNB is present. The class alpha GST overexpression, however, does not seem to be stable if the CDNB resistant cell line is grown for a prolonged period of time in the presence of CDNB. If CDNB is removed after the maximum resistance to the drug is obtained, the B_1 and B_2 subunits remain elevated for at least 3 months (Figs. 3.14 and 3.15).

The relationship of the change in GST subunit expression in CDNB^r and CDNB^r(2) to CDNB resistance in these two cell lines was analysed using the MTT assay. The results are shown in Figures 3.16 and 3.17.

It is apparent from these cytotoxicity assays that resistance to CDNB is not maintained in the CDNB^r(2) cell line. One conclusion that could be drawn from these data, is that it is the overexpression of the B_1 and B_2 subunit which is responsible for the

Figure 3.13 Stability of alpha and pi class subunit overexpression in cells grown in the presence or absence of CDNB, one month after maximum resistance to CDNB had been observed.

Cells were harvested from confluent tissue culture flasks as described in Section 2.1.5 and prepared as described in Section 2.2.1

25μg of soluble protein was subjected to SDS/PAGE, transferred to nitrocellulose and probed with specific antibodies raised against subunits from two known human cytosolic GST classes: alpha (B_1); and pi (π); Stds = Standard (purified protein); WT = wild type NCI H322 cells. $CDNB^r$ = CDNB resistant NCI H322 cells (cultured in the absence of CDNB, after the initial 8 month derivation period). $CDNB^r$ (2) = CDNB resistant NCI H322 cells (cultured in the presence of CDNB after the initial 8 month derivation period.

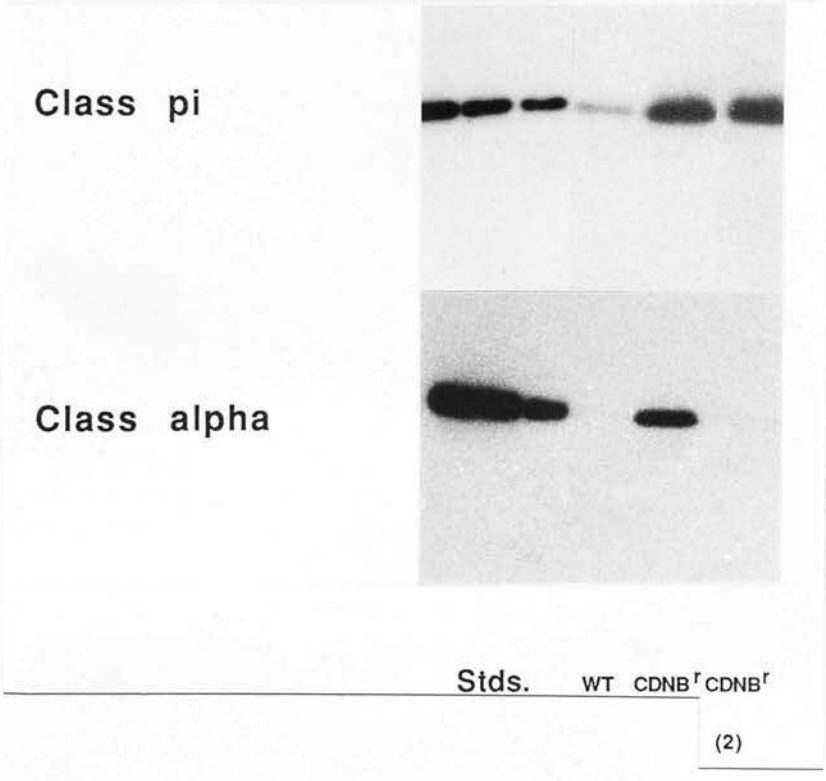


Figure 3.14 Stability of class alpha and pi subunit overexpression in the CDNB^r cell line during length of time in culture.

Cells were harvested from confluent tissue culture flasks as described in Section 2.1.5 and prepared as described in Section 2.2.1

25µg of soluble protein was subjected to SDS/PAGE, transferred to nitrocellulose and probed with specific antibodies raised against known subunits from human alpha and pi class cytosolic GST. Alpha (B_1); pi (π); Stds. = Standard (purified protein); WT = Wild type NCI H322 cells; A to J = CDNB resistant cell line following removal of CDNB.

Time (in days) after CDNB had been removed following the 8 month derivation procedure of CDNB^r:

A = 1 day B = 10 days C = 19 days D = 25 days
E = 31 days F = 35 days G = 35 days H = 39 days
I = 39 days J = 42 days.

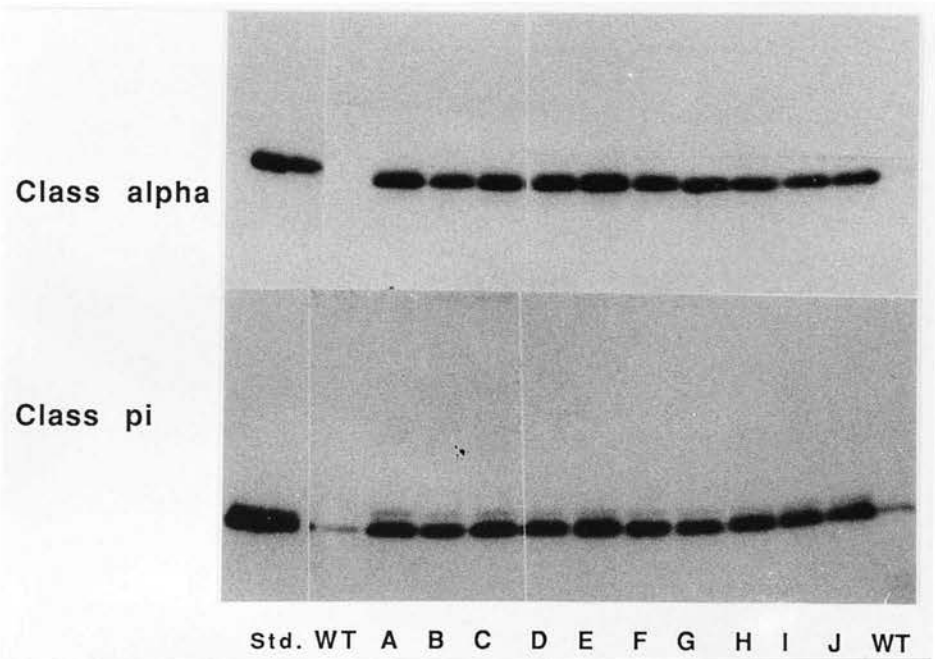


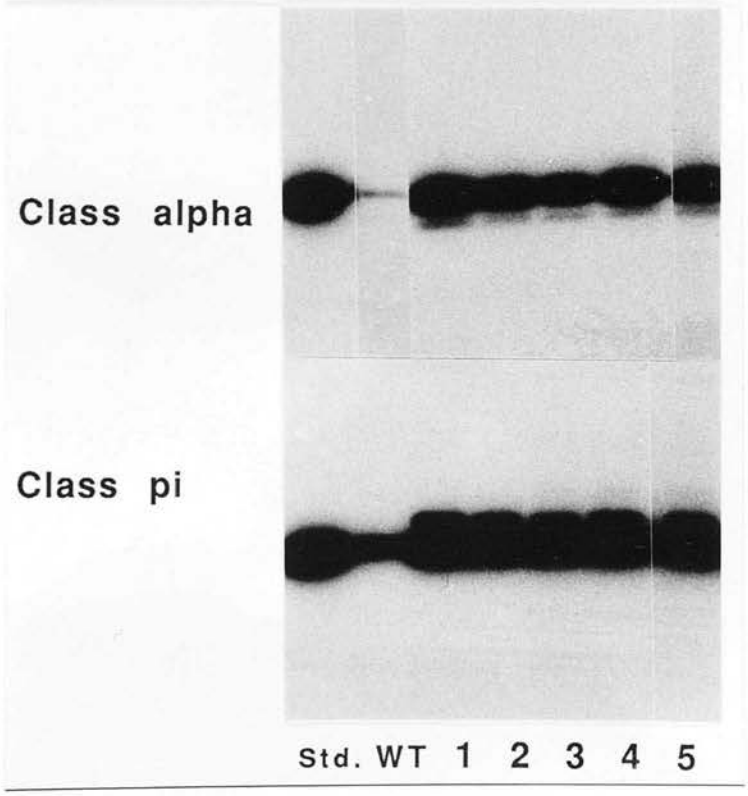
Figure 3.15 Stability of the class alpha and pi subunit overexpression in the CDNB^r line during length of time in culture.

Cells were harvested from confluent tissue culture flasks as described in Section 2.1.5 and prepared as described in Section 2.2.1

25μg of soluble protein was subjected to SDS/PAGE, transferred to nitrocellulose and probed with specific antibodies raised against known subunits from human alpha and pi class cytosolic GST. Alpha (B_1); pi (π); Stds = Standards (purified protein); WT = wild type NCI H322 cells; 1 to 5 = CDNB resistant cell line following removal of CDNB.

Time (in months) after CDNB removed, following the 8 month derivation procedure of CDNB^r:

1 = 1 month 2 = 1.5 months 3 = 2 months 4 = 2.5 months
5 = 3 months



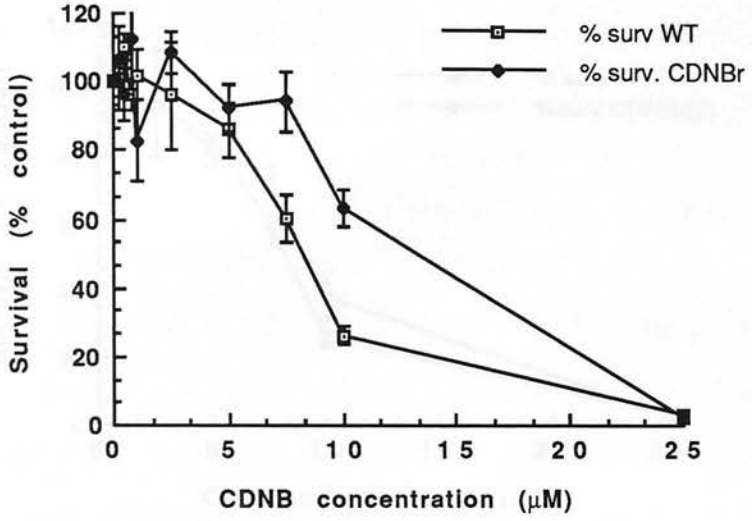


Fig. 3.16 Sensitivity of wild type (WT) and CDNB resistant ($CDNB^r$) cell lines to CDNB.

MTT assays were carried out as described in the Materials and Methods. The values shown are the mean of triplicate determinations.
% surv. = Percentage survival.

$L.D_{50}$ WT = 8.0 μM CDNB.

$L.D._{50}$ $CDNB^r$ = 14.0 μM CDNB.

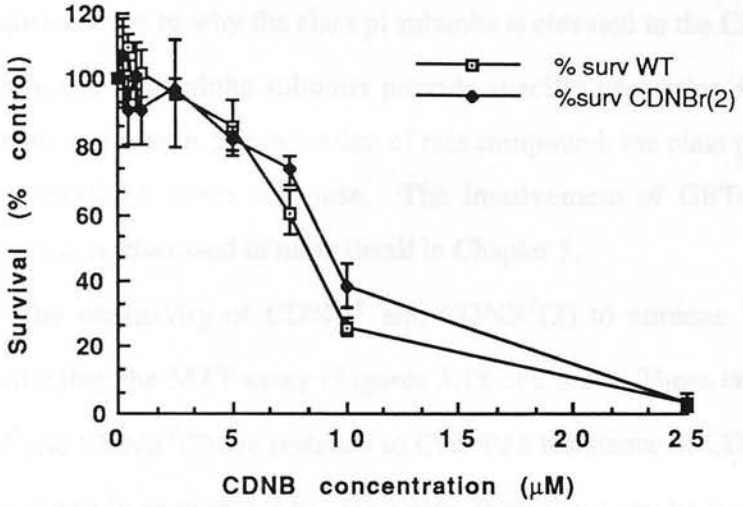


Fig. 3.17 Sensitivity of wild type (WT) and CDNB^r(2) cell lines to CDNB.

MTT assays were carried out as described in the Materials and Methods. The values shown are the mean of triplicate determinations.
% surv. = Percentage survival.

L.D.$_{50}$ WT = 8.0μM CDNB.

L.D.$_{50}$ CDNB^r (2) = 9.0μM CDNB.

observed CDNB resistance in the $CDNB^r$ cell line.

However if these class alpha proteins alone could confer resistance to CDNB, this raises questions as to why the class pi subunits is elevated in the $CDNB^r$ cell line. It may be that while the class alpha subunits provide specific protection from CDNB cytotoxicity through an increase in detoxification of this compound, the class pi subunit is involved in a more generalised stress response. The involvement of GSTs in the stress response phenomenon is discussed in more detail in Chapter 5.

The sensitivity of $CDNB^r$ and $CDNB^r(2)$ to cumene hydroperoxide was also analysed using the MTT assay (Figures 3.18 and 3.19). These analyses indicate that both $CDNB^r$ and $CDNB^r(2)$ are resistant to CHP (the resistance of CDNBr to CHP is discussed in more detail in section 3.2.8). However, from these results it would seem that the class alpha subunits overexpressed in the $CDNB^r$ cell line are only partially responsible for the resistance of this line to cumene hydroperoxide (CHP) since the $CDNB^r(2)$ cell line, which no longer overexpresses B_1 and B_2, is still resistant to cumene hydroperoxide. It is evident from the LD_{50} of $CDNB^r$ and $CDNB^r(2)$ that the latter cell line is slightly more sensitive to CHP in that the LD_{50} of $CDNB^r(2)$ is 66μM CHP and the LD_{50} of $CDNB^r$ is 82.5μM CHP compared to the WT cell line.

This result could be explained by the fact that resistance to CHP may be conferred by both the class alpha and pi GST subunits. However, the class pi subunit has been shown to exhibit little activity towards hydroperoxides and so the results shown in Figures 3.13 and 3.19 taken together, would seem contradictory to this. One explanation for these results could be that the class pi overexpression does not confer resistance to CHP by means of detoxifying this compound, but rather by detoxification of harmful compounds that may be formed due to the action of CHP in the cell.

Whatever the mechanism, the results in Figure 3.13 and 3.19 do suggest that class pi

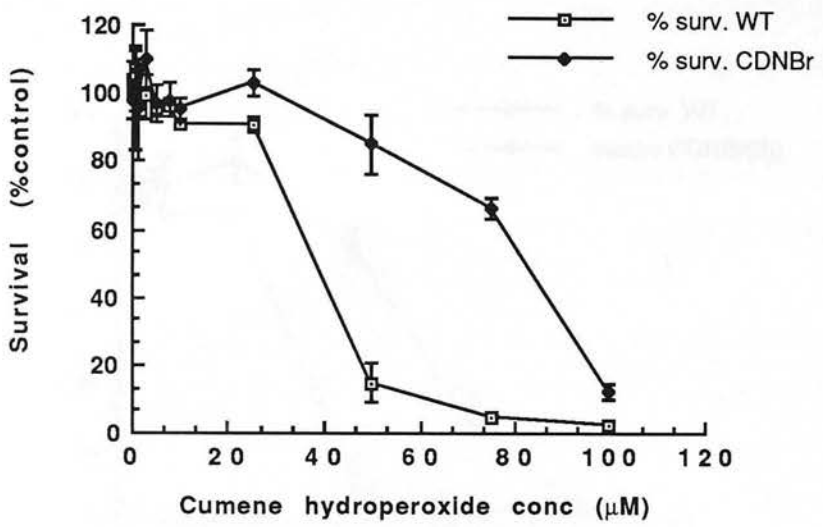


Fig. 3.18 Sensitivity of wild type (WT) and CDNB resistant ($CDNB^r$) cell lines to cumene hydroperoxide.

MTT assays were carried out as described in the Materials and Methods. The values shown are the mean of triplicate determinations.
% surv. = Percentage survival.

$L.D._{50}$ WT = 37.5 μM CHP.

$L.D._{50}$ $CDNB^r$ = 82.5μM CHP.

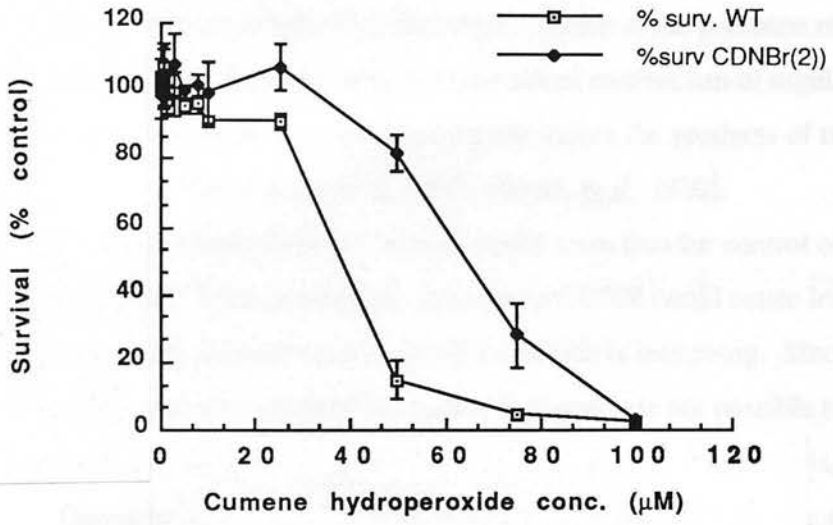


Fig. 3.19. Sensitivity of wild type (WT) and CDNB^r (2) cell lines to to cumene hydroperoxide.

MTT assays were carried out as described in the Materials and Methods. The values shown are the mean of triplicate determinatons.
% surv. = Percentage survival.

L.D.$_{50}$ WT = 37.5μM CHP.

L.D.$_{50}$ CDNB^r (2) = 66μM CHP.

overexpression alone can provide some degree of protection against CHP. The results of Figures 3.13 and 3.18 taken together suggest that protection against CHP is enhanced if class alpha subunits are concomitantly overexpressed with the class pi subunit.

The results seen here with prolonged culture in the presence of CDNB (Figure 3.13) raises several interesting questions as to the actual mechanism of regulation of the B_1 and B_2 subunits. As previously discussed these proteins are the products of two different genes (Tu & Quian, 1986; Rhoads et al, 1987; Hayes, et al, 1990).

From the work presented here it would seem that the control of their expression may be tightly linked. How prolonged exposure to CDNB could cause levels of the B_1 and B_2 subunits to return to those seen in the WT cell line is intriguing. Since it is not known how the protein levels were elevated in the first instance, it is not possible to say what mechanism is responsible for their levels returning to those seen in wild type cells.

One possibility is that during prolonged culture in the presence of CDNB, which has already been shown to be mutagenic to prokaryotes and so could possibly have the same effect on eukaryotes, some regulatory factor common to both B_1 and B_2 becomes mutated. This may in turn cause the levels of B_1 and B_2 to return to those seen in the wild type cell line. Alternatively DNA sequences on the B_1 and B_2 genes that respond to the regulatory factors may themselves become mutated. This would possibly mean that the B_1 and B_2 genes would not longer respond to the regulatory factors and so the genes would be down regulated.

The stability of class alpha and pi subunit overexpression during the length of time of culture of $CDNB^r$ was also analysed (Figures 3.16 and 3.17). It is obvious from this analysis that overexpression of both alpha and pi class subunits is stable for at least three months in the $CDNB^r$ cell line, that is, at least three months after the CDNB has been removed once maximum resistance to CDNB is obtained. As mentioned earlier in this chapter, the $CDNB^r$ cell line was never cultured for longer than three months before the original frozen stocks were returned to and fresh cells were cultured.

3.2.6 Expression of Microsomal GST in CDNB Resistant Cells

GST expression in both nuclear and microsomal fractions was also analysed. The microsomal fraction was in fact a crude membrane extract (Materials and Methods, Section 2.2.2). The pellet obtained during the preparation of cytosolic fractions from cell lines was used in western blotting experiments to investigate the expression of the human microsomal GST in both WT and $CDNB^r$ lines (Figure 3.20). Expression of the microsomal protein was observed in both wild type and $CDNB^r$ cell lines but there was no significant difference in the level of expression in either WT or $CDNB^r$ lines. The Mr of the membrane-bound protein that cross-reacted with the anti-microsomal GST antibody is 14 000 and is distinct from the Mr of the cytosolic enzymes, which range from 24 800 - 27 500 Daltons (Hayes & Mantle, 1986). The expression of the microsomal GST in tumour samples and tumour cell lines is not well documented and so its relevance to drug resistance is unknown.

Recently Clapper & Tew et al (1989) have reported that a rat mammary tumour cell line resistant to chlorambucil expresses a "novel" microsomal GST which is not expressed in the parent cell line. The microsomal GST these authors report as novel is not recognised by antibodies to the human rat microsomal enzyme. However, this "novel" GST has the same Mr as the cytosolic enzymes and it is recognised by antibodies raised against certain rat cytosolic GST subunits. It is feasible that the microsomal fraction prepared in this work is contaminated with cytosolic proteins and that the GST being recognised in the microsomal fraction is a cytosolic enzyme. For this reason more information is required before the protein seen in the microsomal fraction of the chlorambucil-resistant rat mammary cell line can be defined as novel.

3.2.7 Expression of the Human GST subunits in the nuclei of CDNB treated cells

Nuclei were isolated from both WT, $CDNB^r$ and $CDNB^r$ (2) NCIH322 cells (Materials and Methods, section 2.2.3). After the final stage of the isolation procedure, the nuclei fraction was examined microscopically under high power to verify the presence of nuclei. This fraction was then subjected to western blot analysis and the nuclei preparations

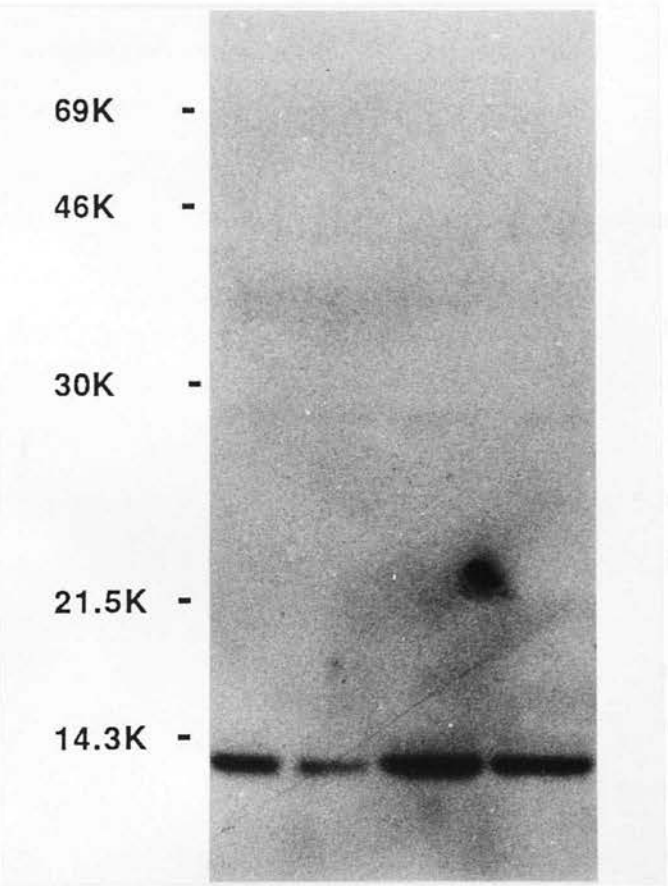
Figure 3.20 The expression of the human microsomal GST in the membrane fraction of WT and CDNB^r cell lines.

Cells were harvested from confluent tissue culture flasks as described in Section 2.1.5 and a membrane fraction prepared according to Section 2.2.2

25μg and 50 μg of soluble protein (as shown below) was subjected to SDS/PAGE, transferred to nitrocellulose and probed with specific antibodies raised to the human microsomal GST.

WT = wild type NCI H322 cells.

CDNB^r = CDNB resistant NCI H322 cells.



probed with antibodies to human class alpha, mu and pi cytosolic enzymes (Figure 3.21).

Only the class pi GST was detectable in the nuclei fraction and there was an apparent increase in the expression of this protein in the nuclei of the CDNB^r (2) line. The significance of this observation is not known. However, it is possible that the elevation of this subunit in the nuclei is a response to the harmful effects of CDNB.

CDNB is a known sulphydryl agent and as such is capable of damaging proteins and so disrupting DNA replication. CDNB has been shown to have mutagenic effects on *Salmonella typhimurium* when subjected to the Ames test (Kerklaan et al, 1987). It is unknown whether this compound can cause DNA damage in eukaryotic cells but it is likely.

CDNB may have various sites of toxicity causing damage to both DNA and proteins. If this compound does have genotoxic effects, it is possible that the apparent elevation in the pi subunit in the nuclei of CDNB^r (2) cells may have occurred in response to DNA damage.

Recent evidence (Tan et al, 1988; Meyer et al 1989) has shown that certain GST isolated from rat liver nuclei have high activity towards DNA peroxides. Although these workers failed to demonstrate the presence of class pi enzymes in the nuclei they isolated, they used rat liver, which does not express pi class GST as an enzyme source. The subunits Tan et al (1988) isolated from rat liver nuclei were 1 and 2 (class alpha), 3 and 4 (class mu) and a subunit homologous but not identical to subunit 5, termed 5*.

In the work presented in Figure 3.21 class alpha and mu enzymes were not detected in the nuclei from either WT or CDNB^r cells. In a study of the response of NCI H322 cells to heat shock, an elevation of the mu class GST subunit was observed in the nuclei fraction of these cells (A. Hall - Personal Communication).

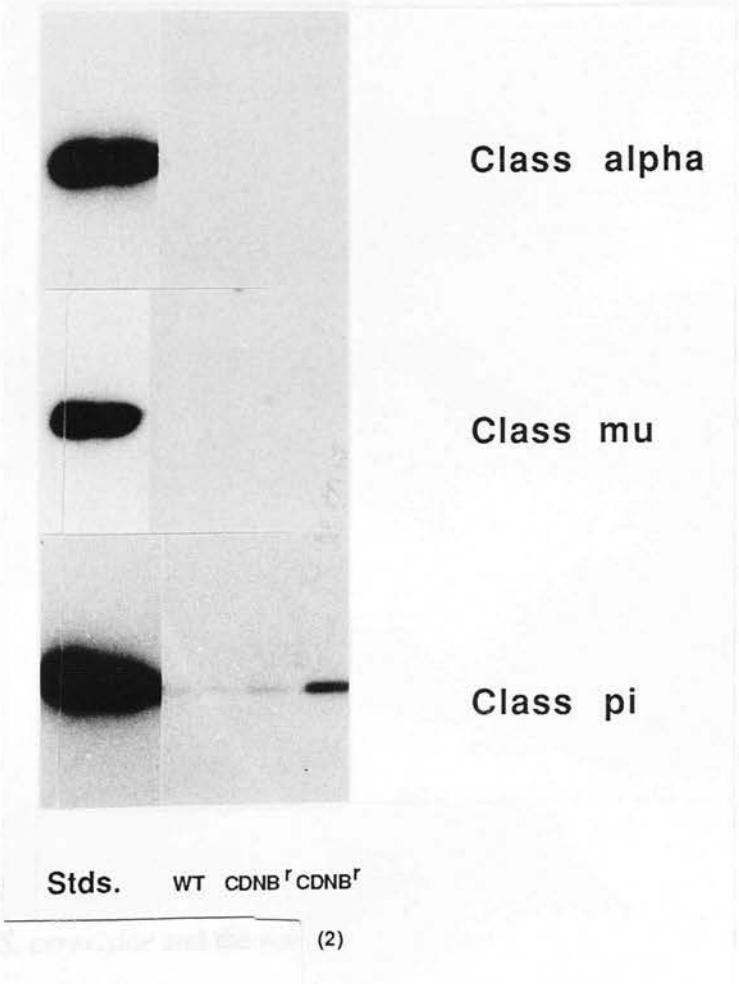
Until the exact mechanism of toxicity of CDNB has been accurately ascertained, it is difficult to state the reason for the overexpression of pi class GST in the nuclei of the CDNB^r (2) cell line. It may be that GST subunits overexpressed in nuclei, in this case the class pi enzyme, are involved in some sort of stress response. This could also possibly account for the observed overexpression of the mu subunit in cells challenged with elevated temperatures (A. Hall - Personal Communication). The role of the GST in a stress response is discussed in more detail in Chapter 5.

Figure 3.21 Expression of human GST classes in nuclei isolated from WT, CDNB^r and CDNB^r(2) cell lines.

Cells were harvested from confluent tissue culture flasks as described in Section 2.1.5. and a nuclei fraction prepared according to Section 2.2.3

50μg of soluble protein was subjected to SDS/PAGE, transferred to nitrocellulose and probed with specific antibodies raised against subunits from the three known human cytosolic GST classes. Alpha (B_1); pi (π) and mu (μ). Stds = Standards (purified protein); WT = wild type NCI H322 cells; CDNB^r = CDNB resistant NCI H322 cells (cultured in the absence of CDNB, after the initial 8 month derivation period).

CDNB^r(2) = CDNB resistant NCI H322 cells (cultured in the presence of CDNB after the initial 8 month derivation period)



The purity of the nuclei fraction isolated in this work is of importance since anomalous results have been obtained by some workers using different types of nuclei isolation procedures(McCuskeret al,1989It is possible that proteins from the cytosol may have been co-isolated with the nuclei so that what is possibly seen in Figure 3.21 is cytosolic GST contamination.

3.2.8 Cross Resistance Patterns of the CDNB resistant cells

The cross resistance of the $CDNB^r$ to other toxic compounds was also examined. The results from the analysis using cumene hydroperoxide are shown in Figure 3.22. As can be seen, $CDNB^r$ is approximately 2.5-fold resistant to cumene hydroperoxide compared to the wild type line as ascertained from the LD_{50}'s.

Cumene hydroperoxide is an organic hydroperoxide and a diagnostic substrate for both selenium-dependent and selenium-independent glutathione peroxidase. It is a highly toxic compound since it acts as a peroxidising agent. Lipid peroxidation is a complex chain reaction which can cause detrimental effects to cellular membranes and releases toxic breakdown products which may then go on to damage other important cellular macromolecules (Slater 1984; Ketterer et al, 1987).

Examples of some breakdown products of lipid metabolism are compounds such as lineoleate hydroperoxide and arachidonate hydroperoxide. These substances have also been shown to be good substrates for the class alpha GSTs (Tan et al, 1984; Ketterer et al, 1987) and it is postulated that detoxification of lipid peroxides by GST is one mechanism of protection from potential cellular damage by the peroxides (Ketterer et al, 1987). This hypothesis is supported by the evidence from experiments carried out with cumene hydroperoxide during this project.

It has been shown in the work presented in this thesis that the class alpha GST subunits (B_1 and B_2) are overexpressed in the $CDNB^r$ cell line (see Figures 3.10 and 3.11) and that as well as exhibiting resistance to CDNB, this cell line is also resistant to cumene hydroperoxide (Figure 3.22). Supportive evidence that the class alpha subunits overexpressed in the $CDNB^r$ line protect against the damaging effects of cumene hydroperoxide comes from the work of Black et al (1990) in which the B_1 subunit was expressed in *S. cerevisiae* and the sensitivity of recipient cells to a range of compounds

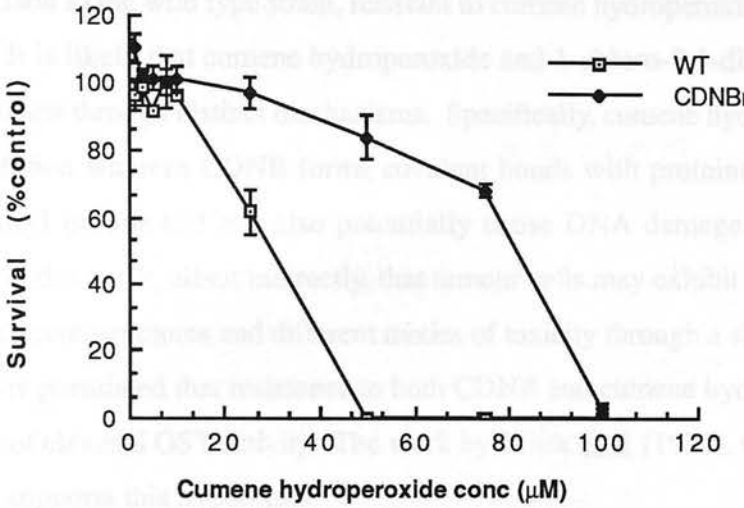


Fig. 3.22 Sensitivity of wild type (WT) and CDNB resistant (CDNBr) cell lines to cumene hydroperoxide.
MTT assays were carried out as described in the Materials and Methods. The values shown are the mean of triplicate determinations.

$L.D._{50}$ WT = 30.0μM CHP.

$L.D._{50}$ CDNBr = 82.5μM CHP.

was examined. It was found that the yeast strain expressing the B_1 subunit was, by comparison to the wild type strain, resistant to cumene hydroperoxide.

It is likely that cumene hydroperoxide and 1-chloro-2,4-dinitrobenzene cause their toxic effects through distinct mechanisms. Specifically, cumene hydroperoxide leads to lipid peroxidation whereas CDNB forms covalent bonds with proteins through reactions with sulphydryl groups and can also potentially cause DNA damage. However, it has been shown in this work, albeit indirectly, that tumour cells may exhibit resistance to compounds with different structures and different modes of toxicity through a single mechanism. In this case, it is postulated that resistance to both CDNB and cumene hydroperoxide has arisen as a result of elevated GST activity. The work by Black et al (1990), which is of a more direct nature, supports this hypothesis.

The situation regarding the $CDNB^r$ cell line may be analogous to that seen in other mammalian tumour cell lines resistant to a range of toxic anticancer agents. However, in these drug resistant models the structures and mechanisms of lethality of the antitumour drugs are more diverse than those of the compounds discussed above. As stated in the Introduction, the routes by which tumour cells exhibit resistance to these anticancer agents is the subject of continuing intensive research but the emerging picture is that no one mechanism is solely responsible for all instances of resistance to a single drug. It is becoming evident that resistance is multifactoral. It is possible that the GSTs due to their broad substrate specificities may be an important factor in the resistance process to several classes of drugs.

In this piece of work it was assumed that if the GST play a causal role in drug resistance, then theoretically the $CDNB^r$ cell line, which overexpresses both class alpha and pi subunits, would exhibit cross resistance to some of the anticancer agents commonly associated with this process.

The results of cytotoxicity assays using a range of antitumour drugs are shown in Figures 3.23 to 3.26. It is evident that in this $CDNB^r$ model, the overexpressed GST subunits are not conferring resistance to the drugs analysed. Although initially discouraging and seemingly contradictory to certain results obtained by other workers (Manoharan et al, 1987; Puchalski et al, 1989; Black et al, 1990) it is quite possible that given the

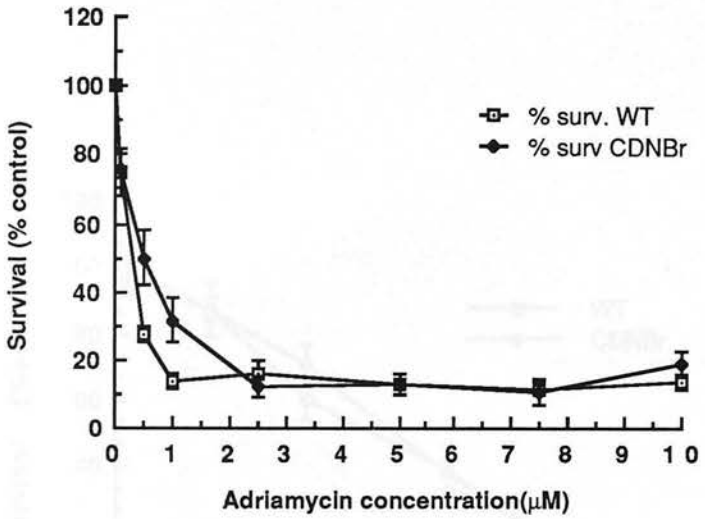


Fig. 3.23 Sensitivity of wild type (WT) and CDNB resistant ($CDNB^r$) cell lines to adriamycin.

MTT assays were carried out as described in the Materials and Methods. The values shown are the mean of triplicate determinations.

% surv. = Percentage survival.

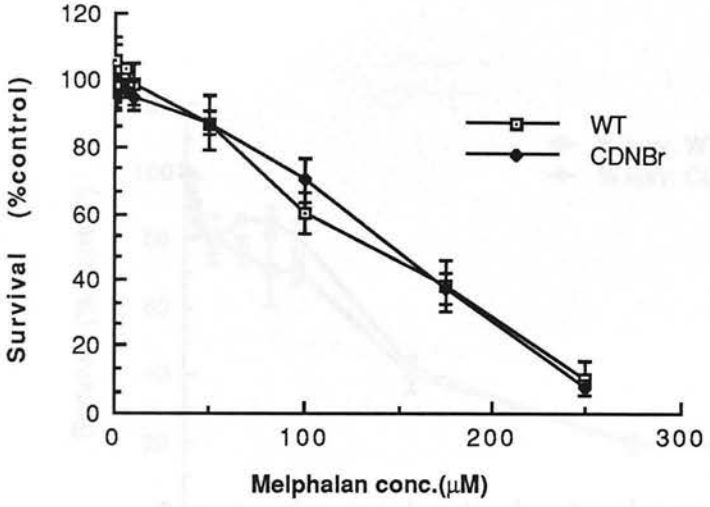


Fig 3.24 Sensitivity of wild type (WT) and CDNB resistant (CDNBr) cell lines to melphalan.

MTT assays were carried out as described in the Materials and Methods. The values shown are the mean of triplicate determinations.

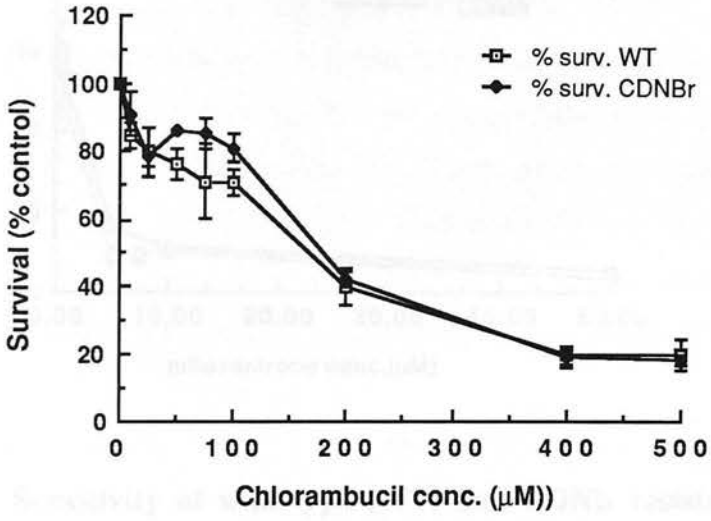


Fig. 3.25 Sensitivity of wild type(WT) and CDNB resistant ($CDNB^r$) to chlorambucil.

MTT assays were carried out as described in the Materials and Methods. The values shown are the mean of triplicate determinations.

% surv. = Percentage survival.

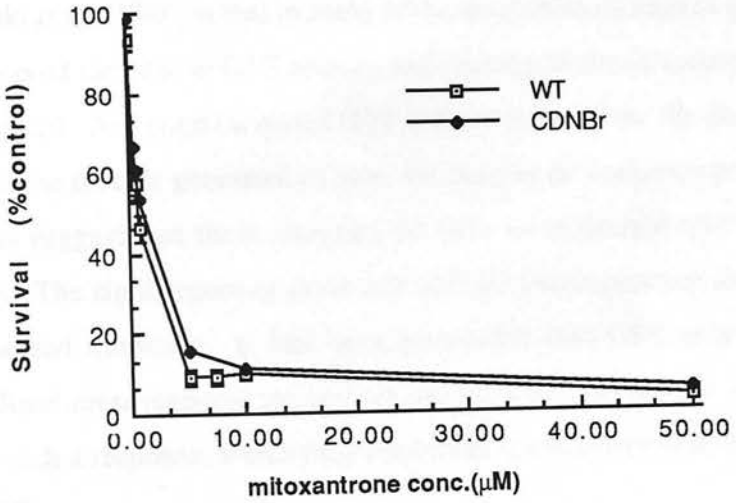


Fig 3.26 Sensitivity of wild type (WT) and CDNB resistant (CDNBr) cell lines to mitoxantrone.

MTT assays were carried out as described in the Materials and Methods. The values shown are the mean of triplicate determinations.

the multifactoral nature of drug resistance, other necessary proteins were not co-induced with the GSTs in this particular cell line. Unfortunately, it was outwith the scope of this project to investigate other cellular factors thought to be involved in the drug resistance.

Another possibility which again is in disagreement with Black et al (1990) and Puchalski et al (1989) is that in many of the drug resistant models generated (see Table 1.5), the observed increase in GST activity seen in drug resistant variants relative to parent lines, is incidental. Although increased GST activity may not be the direct cause of resistance in many of the models generated to date, the number of workers reporting this kind of finding tends to suggest that these enzymes do have an important role to play in the resistance process. The significance or exact role of GST overexpression in these models remains an unanswered question. It has been postulated that GST may be involved in a more generalised stress response mechanism and that challenging cells with toxic anticancer drugs elicits such a response, which may contribute to resistance (Hayes & Wolf, 1988; Lewis et al, 1988).

Whatever the role the GSTs have in drug resistance, it is clear from the work presented in this thesis that in the particular model generated during this project the GST subunits overexpressed in the CDNBr cell line do not confer resistance to the anticancer drugs analysed in Figures 3.23 to 3.26. The overexpressed subunits may however confer resistance to CDNB and cumene hydroperoxide showing that these proteins can afford protection against certain cytotoxic compounds of dissimilar structure and functon.

It is evident from the work of others (Manoharan et al, 1987; Puchalski et al, 1989; Black et al, 1990) that GSTs do have the potential to confer resistance to certain compounds such as adriamycin and chlorambucil. Based on evidence of this nature and further work showing that certain anticancer drugs can act as substrates for different GST subunits (Dulik et al, 1986; Smith et al, 1989), it would seem that GSTs can confer resistance to a number of antineoplastic drugs. It is therefore likely that in many of the models referenced in Table 1.5, the increased GST activity that is observed is at least partially responsible for the resistance to the drugs described.

As drug resistance can be conferred by a number of mechanisms, it is important that future analysis of resistant cell lines is conducted in such a way as to identify as many resistance mechanisms as possible in a given cell line. At present this is seldom achieved in

the majority of analyses of this nature.

Another unanswered question in the field of drug resistance is just how the resistant population arises. It could be due to a sub population of cells that already expresses the factors that confer resistance and which is selected out in the presence of drug concentrations that are toxic to the majority of cells in the population. Alternatively, in response to toxic drug concentrations, cells may have the capacity to induce the factors which confer resistance.

In the $CDNB^r$ model discussed here, it is not possible to say how the cellular population arose which overexpressed both elevated pi and alpha class subunits. The mechanism by which these subunits were elevated was, however, addressed.

3.2.9 Molecular Basis for the Alpha Class GST Overexpression

The work of several investigators has shown that GST overexpression in various drug resistant models can occur via several routes. For example, the adriamycin resistant MCF-7 cell line has been analysed with respect to mechanism of class pi overexpression (Cowan et al, 1986; Batist et al, 1986). In this cell line the class pi subunit is elevated at least 45-fold. This change in protein level has been shown to be accompanied by an increase in the mRNA coding for this subunit. However, no increase in gene copy number has been observed. These results indicate that the elevation seen at the protein level occurs either by an increase in transcription rate or by a stabilisation of the pi mRNA.

In the work by Robson et al (1986; 1987), the Chinese hamster ovary cell line resistant to chlorambucil showed a marked increase in the Yc class alpha subunit. Upon examination, there was found to be an increase in both the mRNA levels and gene copy number as determined by northern and Southern blot analysis respectively (Lewis et al, 1988). The protein over-expression therefore appears to be a result of an increase in transcription due to gene amplification.

To examine whether the overexpression of alpha and pi class GST is due to transcriptional activation, gene amplification or protein stabilisation Southern and northern blot analyses were performed on both the WT and $CDNB^r$ lines. The Southern blots

(Figures 3.27 and 3.28) indicate that the overexpression of both the alpha and pi class subunits is not a consequence of gene amplification.

Northern blot analysis (Figure 3.29) indicate that the increase in the levels of the B_1 and B_2 subunits is not accompanied by a notable elevation of mRNA levels encoding these GST.

It is possible therefore, that the alpha class subunits have been stabilised and so their turnover rate is slower than that seen in the WT cell line. Alternatively, there may have been an increase in the rate of translation of protein from the mRNA.

Other workers (McClarty et al, 1988) investigating the protein ribonucleotide reductase in a hydroxyurea resistant mouse cell line, have shown overexpression of two protein subunits (M1 and M2), which are responsible for the activity of the enzyme. This overexpresson at the protein level could be attributed to increases in the levels of mRNA for both subunits when the cells were cultured in the presence of hydroxyurea up to a certain concentration. However, when concentrations of the drug were increased still further, there was a further elevation of the M1 and M2 subunits. This was not accompanied by further increases in the mRNA levels. Pulse chase experiments indicated that the half lives of both polypeptides were increased approximately two-fold. This could be accounted for by protein stabilisation. .

The information presented in Figures 3.27 to 3.29 taken together with the work discussed above (Cowan et al, 1986; Batist et al, 1986; Robson et al, 1986; Robson et al, 1987; Lewis et al, 1988) illustrate that GST overexpression may occur via a variety of routes. The factors that determine which mechanism is operational remain to be elucidated and are far from obvious. However, the type of drug to which cells are exposed as well as the length of time of exposure are likely to be important determinants. As discussed earlier, the tissue of origin of the tumour cells may determine whether transacting factors are present or absent and hence influence the ultimate mechanism responsible for GST overexpression.

Regulation of GST expression has received much attention in recent years (Ding & Picket, 1985; Picket, 1987; Daniel et al, 1987; Cowell et al, 1988; Telahowski-Hopkins et al, 1988). However, this work has focussed predominantly on regulation of GST expression in the rat. Whether parallels can be drawn between regulation of the rat and human GST genes remains to be established.

Figure 3.27 Southern blot analysis of WT and CDNB^r cell lines probed with a cDNA to a human alpha class subunit.

DNA was isolated from cells as described in Section 2.6.1

20µg of DNA was digested with (A) EcoRI, (B) Bam HI and (C) Pst I., separated by electrophoresis on a 1% agarose gel, transferred to hy-bond^{TM} membrane and hybridised with a nick translated [^{32}P] labelled full length cDNA clone (Board & Webb,1987)
Liv = control human liver DNA
WT = DNA from wild type NCI H322 cells.
CDNB^r = DNA from CDNB resistant NCI H322 cells.

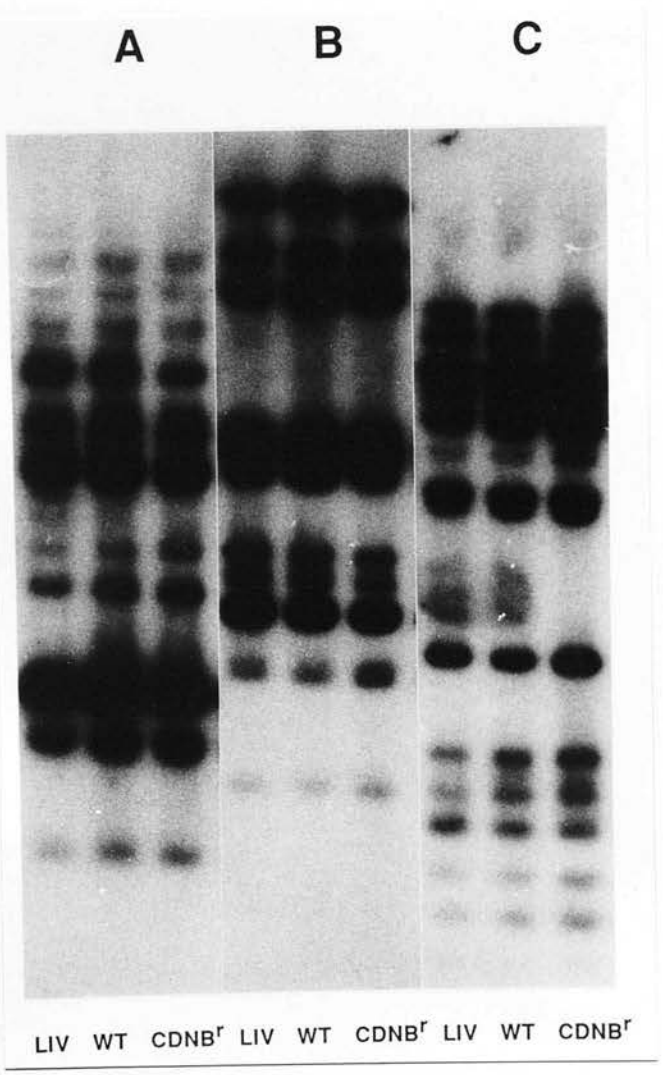


Figure 3.28 Southern blot analysis of WT and CDNB^r cell lines probed with a cDNA to the human pi subunit.

DNA was isolated from cells as described in Section 2.6.1

20µg of DNA was digested with (A) Eco RI, (B) Bam HI and (c) Pst I, separated by electrophoresis on a 1% agarose gel, transferred to hy-bond^{TM} membrane and hybridised with a nick translated [^{32}P] labelled full length cDNA clone (Kano et al, 1987)
Liv = control human liver DNA.
WT = DNA from wild type NCI H322 cells.
CDNB^r = DNA from CDNB resistant NCI H322 cells.

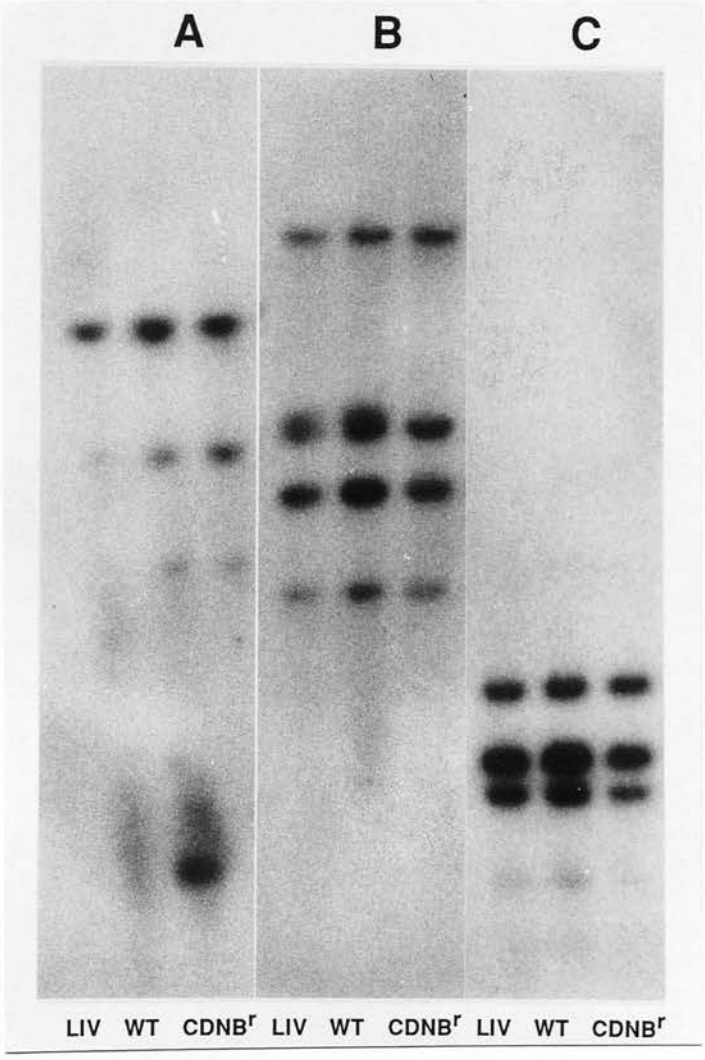
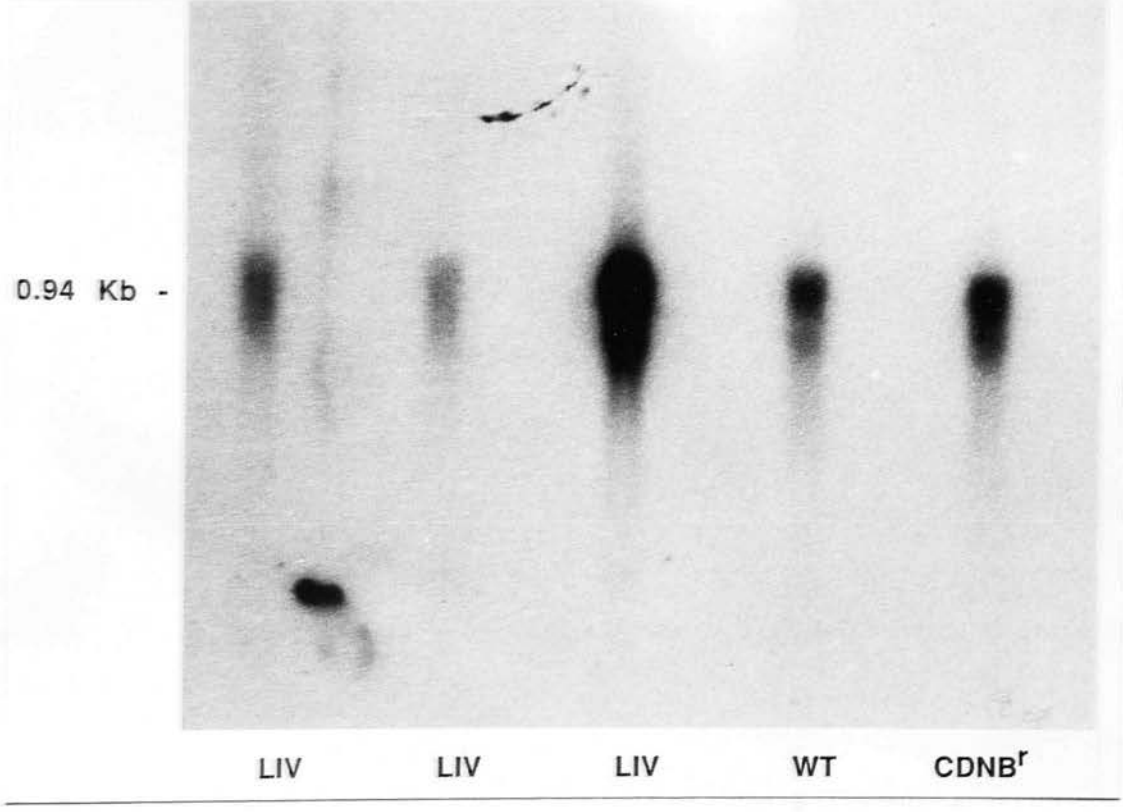


Figure 3.29 Northern blot analysis of WT and CDNB^r cell lines probed with a cDNA to a human alpha class subunit.

RNA was isolated from cells as described in Section 2.6.2

15µg (WT and CDNB^r) and 5, 10 and 15µg (liv) was electrophoresised on a 1.5% agarose gel, transferred to hy-bond^{TM} and hybridised with a nick translated [^{32}P] labelled full length cDNA clone (Board & Webb, 1987).
liv = control human liver RNA
WT = RNA from wild type NCI H322 cells
CDNB^r = RNA from CDNB resistant NCI H322 cells



A model for mechanism of regulation of a rat Ya gene is shown in Figure 1.4. It has been shown that planar aromatic compounds such as 3-methylcholanthrene and phenobarbital can induce the rat liver Ya subunit (Telakowski-Hopkins 1988). The proposed mechanism of induction, as outlined in Figure 1.4 , is that the planar aromatic compound binds to the dioxin receptor forming a complex. The ligand receptor complex is then translocated to the nucleus. Here it causes transcriptional activation of the Ya gene by interacting with a regulatory sequence that is known to be responsive to planar aromatic compounds (see Section 1.2.8 (ii)). This is only one of several proposed mechanisms of action.

Whether other compounds, such as antineoplastic drugs cause elevation of GST subunits via mechanisms similar to the one outlined above is not known. However, it is an area of research which must surely yield exciting results.

Whether factors that are involved in the control of GST expression are also involved in regulation of other resistance mechanisms awaits clarification. It is noteworthy however, that oncogenes have been shown to increase expression of both GST pi and P-glycoprotein concomitantly (Burt et al 1988).

Further evidence is necessary before conclusions regarding coordinated regulation of resistance mechanisms can be reached. The data available suggests that certain proteins involved in resistance can be co-induced but it depends on the model.

3.3.0 CONCLUSION

The data presented and discussed above are in agreement with the results presented by a number of workers (Black et al, 1990). In recent years, a growing number of reports have appeared in the literature of increases in GST subunit expression in cells challenged and made resistant to cytotoxic antitumour agents.

As more has become known of the GST and their biochemical characteristics, attention has focussed on a potential role for the GST in the drug resistance phenomenon. Supportive evidence for this role has come from results showing that when cDNAs coding for certain GST subunits are transfected into eukaryotic cells, resistance to certain agents is conferred (Black et al, 1990). Further to this, results have been presented showing both melphalan and BCNU can act as substrates for certain GSTs (Dulik et al, 1986; Smith et al,

1989).

From the available evidence, it seems that the GST may have the potential to confer resistance to cytotoxic drugs as a result of the GST catalysed formation of drug-glutathione conjugates. However, much of the work in this field has involved derivation of cell lines resistant to antitumour agents and measurement of overall GST activity. Changes in individual subunits and ability of these subunits to catalyse detoxification of the drug are seldom investigated. Many of these cell lines have also exhibited cross resistance to other anticancer drugs and GSTs have been speculated to be involved in this phenomenon as a result of their broad range of substrate specificities.

Since the compound used to generate the CDNB^r cell line discussed in the preceding Chapter, is a known GST substrate, it is very likely that the CDNB resistance demonstrated by this line, is a direct result of elevation of the class alpha and pi GST subunits. That resistance to cumene hydroperoxide is also observed, suggests that GSTs do indeed have the capacity to confer resistance to compounds with different mechanisms of action.

CHAPTER 4

CLASS PI GLUTATHIONE S-TRANSFERASE EXPRESSION IN LEUKAEMIA CELLS

4.1.0 INTRODUCTION

Although tumour cell models have been used predominantly to investigate the possible mechanisms involved in the drug resistance phenomenon, their relevance to the clinical situation is uncertain. Work comparing the expression of P-glycoprotein mRNA in normal and tumour tissue has sometimes yielded ambiguous results (Bell et al, 1985;Ma et al 1987) and it has become obvious that P-glycoprotein is not the only factor involved in this process. If chemotherapy drug regimens are to prove effective, the underlying mechanisms responsible for drug resistance must be elucidated in clinically significant tumour populations.

The information obtained from resected tumours is perhaps questionable. Solid tumour masses consist of a heterogenous cell population and the cells in the middle of a tumour mass are likely to differ considerably from those cells on the periphery. For example, cells within a single tumour will receive markedly different levels of blood supply and it is reasonable to assume that the oxygen and nutrient supply are likely to be more

accessible to those cells on the periphery of the tumour than those in the centre of the tumour mass.

Much of the work carried out on tumour samples has involved homogenising pieces of resected tumour tissues that may consist of cells from various regions of the tumour. The results from analyses of homogenised tumour tissue are difficult to interpret if one considers that different tumour cell types are mixed during the preparative procedures.

It may therefore prove that haematological cancers provide a more homogenous model with which to work. This kind of malignancy has the advantage that it can be separated into different cell populations and these can then be analysed individually.

4.2.0 RESULTS AND DISCUSSION

In this study, class pi GST expression in human leucocytes was investigated for a series of haemopoietic malignancies and compared with levels found in peripheral leucocytes of normal control subjects. Nine patients with myelodysplastic syndrome (MDS), a pre-leukemic state, 21 patients with acute myeloblastic leukemia(AML) [5 treated, 5 secondary and 11 relapsed or refractory patients] and 32 patients with chronic lymphocytic leukemia (CLL)[7 untreated and 25 treated] were analysed.

The levels of P-glycoprotein mRNA expression had previously been investigated in the same samples that were analysed in this study (Holmes et al, 1989; Holmes et al, 1990b). From these previous results it is apparent that although P-glycoprotein expression is elevated in some leukemia samples, it is not in others. This raises questions as to the role of P-glycoprotein in the drug resistance process. Although it appears from the work of Holmes et al (1989; 1990b) that P-glycoprotein may be involved indrug resistance in some instances, it is also obvious that other mechanisms may be operational in other instances. For this reason, GST pi expression was examined in the same samples previously analysed by Holmes et al (1989; 1990b).

As described in the Materials and Methods section, RNA slot blot analysis was used to investigate mRNA levels of class pi GST in both leukaemic and normal control leucocytes. The RNA was quantified using densitometry readings and normalised for any loading differences using readings obtained from actin RNA densitometry measurements on

the same samples (Holmes et al, 1990a).

All the measurements obtained from the leukaemia samples were compared to the measurements obtained from total peripheral leucocytes collected from ten normal control subjects (4 men, 6 women.) ranging in age from 23 to 83 .The lowest densitometry measurement from this panel of normal leucocytes was designated as being 1 arbitary unit. The level of class pi mRNA expression ranged from one to three in these normal samples (Table 4.1). Leukaemia samples showing RNA levels of 4 arbitary units or more were classed as having elevated GST pi expression (Table 4.1). Of the patients with MDS, a low grade neoplastic proliferation of haemopoietic stem cells which often develops into acute myeloblastic leukaemia, 8 out of 9 patients showed increased expression of class pi mRNA, the range being 4-10 arbitary units.These patients were all treated with intermittent or continuous low dose chlorambucil.

Of the 21 patients with acute myelodysplastic leukemia (AML), 12 patients showed elevated class pi mRNA expression. Of these, 4 out of 5 were untreated patients (6-8 units), 1 out of 5 was a secondary AML (ie. secondary to previous chemotherapy and radiotherapy for carcinoma of the breast; 6 units) and 7 out of 11 were patients with relapsed or refractory AML (4-24 units).

Thirty two patients with chronic lymphocytic leukemia (CLL) were also analysed. Of 7 untreated patients, 3 showed elevated class pi GST mRNA levels (5-27 units) while 21 out of 25 treated CLL patients showed increased class pi GST mRNA expression (4-28 units). Figure 4.1 shows representative examples of MDS, AML and CLL showing elevated levels of GST pi mRNA compared to normal control leucocytes. The loadings of the RNA on these blots was standardised against actin RNA from the same samples.(results not shown)

The relevance of this data to drug resistance is not known neither are the mechanisms whereby GST pi mRNA levels are elevated. This could be attributable to gene amplification, mRNA stabilisation, an increase in mRNA transcription or any combination of these.

In addition to the actual mechanism of mRNA overexpression, the cellular events leading to induction/overexpression are also unknown. It is possible that some event during the development of leukemia results in class pi GST overexpression in certain instances. It

TABLE 4.1 GST Pi overexpression in Leukaemia

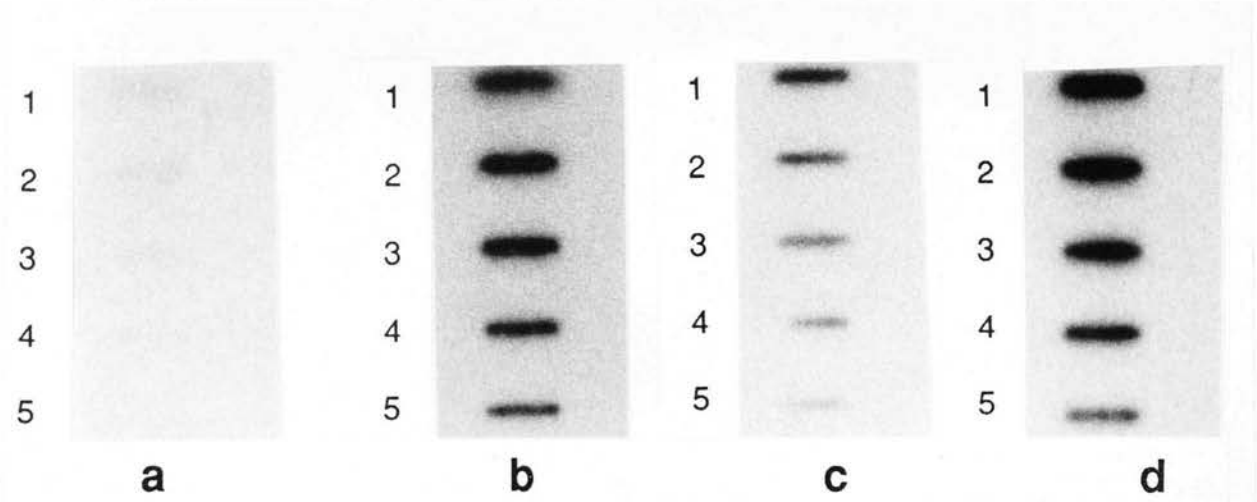
NORMAL CONTROLS	RELATIVE LEVELS OF GST PI	
Total peripheral leucocytes (n=10)	1 - 3	
	No. of Patients with increased expression of GST pi in mRNA	(range)
MYELODYSPLASTIC SYNDROME (MDS) (n=9)	8	(4 - 10)
ACUTE MYELOBLASTIC LEUKAEMIA (AML) (n=21)		
Untreated (n=5)	4	(6 - 8)
Secondary (n=5)	1	(6)
Relapsed or refractory (n=11)	7	(4 - 24)
CHRONIC LYMPHOCYTIC LEUKAEMIA (CLL) (n=32)		
Untreated (n=7)	3	(5 - 27)
Treated (n=25)	21	(4 - 28)

Figure 4.1 GST pi overexpression in MDS, AML and CLL samples

Representative RNA slot blots from (a) Normal leucocytes and patient with (b) MDS (c) AML and (d) CLL.

One arbitary unit represents the level of GST pi expression demonstrated in normal leucocytes.

1-5 = Doubling dilutions of RNA.



has already been shown that some oncogenes can lead to induction of both pi class mRNA and P-glycoprotein mRNA in cells which are transfected with the ras oncogene (Li et al,1988) and oncogenes are known to be aberrantly expressed in cancer cells.

Class pi GST expression has received much attention in recent years since it has become evident that it can be used as a marker for preneoplastic lesions in rat liver cells. It is hypothesised that exposure to carcinogens results in altered rat liver foci that overexpress a range of phase II drug detoxification enzymes including class pi GST (Farber, 1984). Since certain cancers are known to result from carcinogen induced changes it is possible that cancer cells may already be selected for increased class pi GST expression. This may in turn provide further protection from challenge with other cytotoxic compounds such as antitumour agents. This is, however, hypothetical and awaits clarification but it is interesting to note that the lymphoblastic cell line CEM (derived from a leukaemia) expresses high GST pi (9 units) relative to normal leukocytes (Table 4.1).

There are also many other examples in the literature where class pi GST is found to be overexpressed in tumour tissue relative to normal surrounding tissue and it is possible that this is a result of the carcinogenesis process (Soma et al, 1986; Di Ilio et al, 1988; Moscow et al, 1989).

Other workers looking at haemopoietic malignancies have also shown increases in GST pi expression in the leucocytes of leukaemia patients compared to normal control leucocytes (McQuaid et al, 1989; Moscow et al, 1989). However, although there are now many examples in the literature of increases in class pi GST expression in tumour tissue relative to normal surrounding tissue, the relevance of this to drug resistance is unclear and the information available largely circumstantial.

It is necessary to show that class pi GST can lead to resistance by detoxifying the anticancer drugs to which resistance is exhibited or by detoxifying metabolites produced by the action of the antitumour agents. Until this information is available, the results in this field of active research will remain circumstantial.

There is relatively little information available on haemopoietic malignancies but the data emerging from work on other tumour types shows that class pi GST is generally elevated in tumours derived from colon (Kodate et al, 1986; Moscow et al, 1989; Howie et al, 1990) stomach (Tsutsume et al, 1987; Howie et al, 1990) and lung (Di Ilio et al, 1988;

Moscow et al, 1989; Howie et al, 1990). It is interesting that lung, colon and gastric tumours derive from tissues that are associated with increased carcinogen exposure and it is possible, as discussed previously, that increases in class pi GST levels may reflect carcinogen induced changes similar to those seen in rat hyperplastic nodules.

As stated earlier, the samples analysed in this study had already been examined with respect to their mdr 1 mRNA levels of expression (Holmes et al, 1989; Holmes et al, 1990b). The results from the mdr 1 mRNA work and the work presented here were compared. Little correlation between class pi GST and mdr 1 mRNA levels in these leukemia samples was seen. This is shown graphically in Figure 4.2. Using Spearman's Rank order correlation, non-significant negative correlation was found between mdr 1 and class pi GST expression in both MDS ($r = -0.412$) and AML specimens ($r = -0.314$). Positive correlation between mRNA expression of these two proteins was seen in the CLL samples. However the correlation was weak ($r = 0.335$, $p = 0.05$ two tailed test).

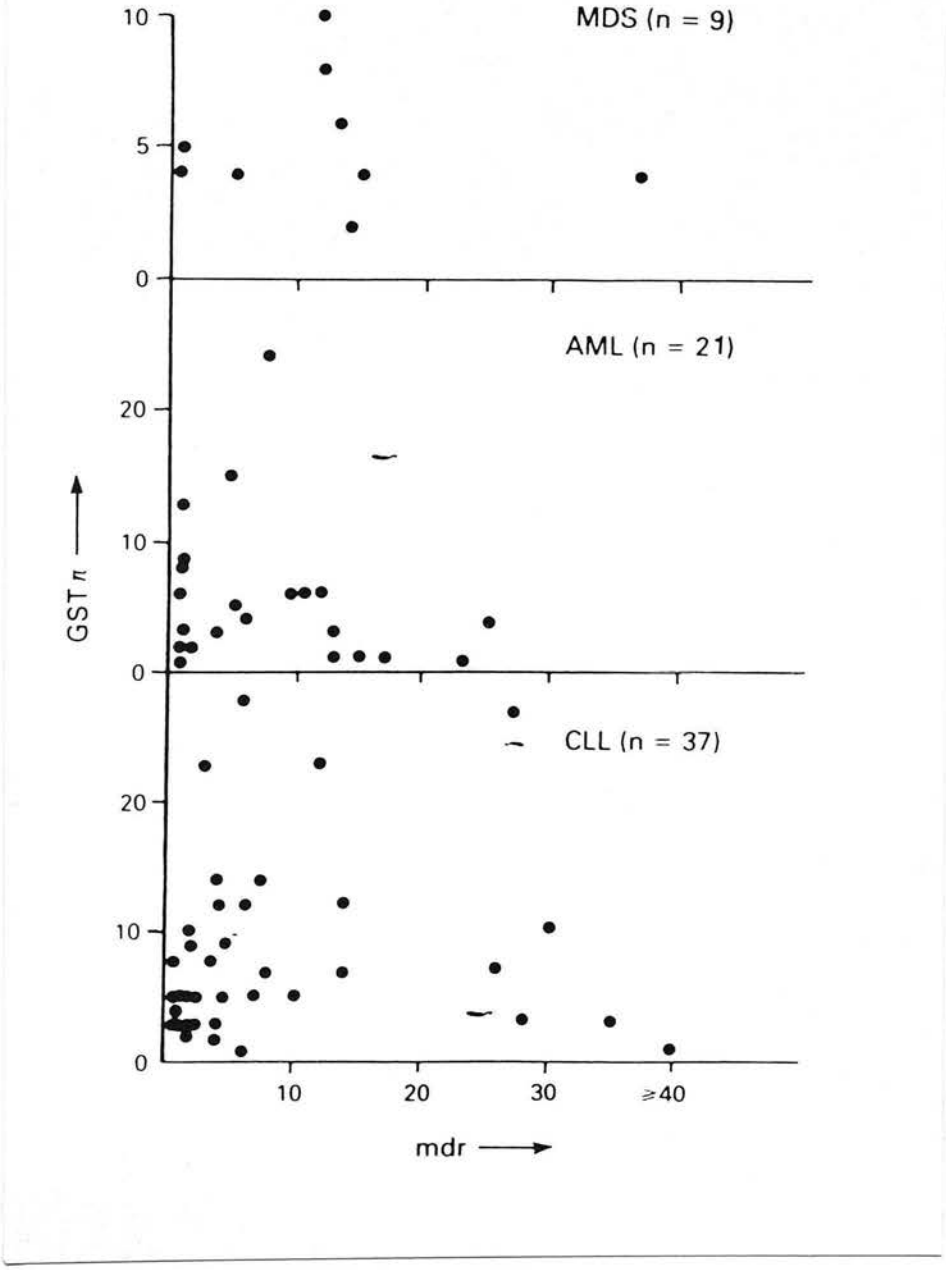
It is also evident in these leukaemia samples, that other mechanisms of resistance may also be operational in certain intances as there are those samples which express neither protein. Recently, there has been a report of low levels of DNA topoisomerase II in CLL (Potmesil et al, 1988). These workers suggested that this may be a clinically significant route to a drug resistant state for those tumours with a large population of non-proliferating cells.

The picture with regards to resistance to anticancer agents is becomining increasingly complex. That it is a multifactional process is obvious. However, the factors that determine which mechanisms operate in a tumour population at a given time are less clear. This is but one of the many challenges to workers in this field.

Although the work discussed above detailing mRNA levels of the class pi GST expression in human leukemia samples provides informative data, it is also necessary to investigate the protein levels of class pi GST since ultimately it is the proteins that establish the phenotype of cells by their ability to catalyse chemical reactions.To this end leucocyte samples from leukaemia patients (all AML) of Professor Bob Souhami and normal healthy volunteers were compared using the western blot technique.

At the time the Western blot analysis was carried out, the origin of each sample was unknown. However, this information was revealed upon completion of this analysis. The

Figure 4.2 **Comparison of GST pi and mdr 1 mRNA levels in MDS, AML and CLL samples using Spearman's Rank Order correlation.**



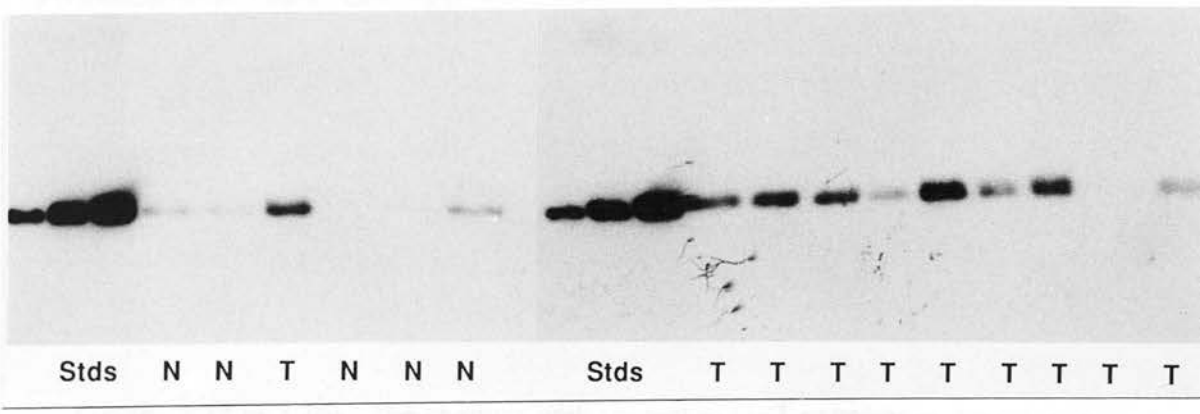
results are shown in Figure 4.3. As can be seen, the class pi GST protein is detectable in the majority of the samples but it is evidently expressed at higher levels in the leukaemia samples. No class alpha or mu protein was detectable in any of the samples analysed (results not shown).

These data are consistent with the results presented above with respect to class pi GST mRNA overexpression and the discussion in relation the overexpression of pi mRNA in leukaemia samples is also applicable to the results presented in Figure 4.3. However, it should be noted that where possible DNA, RNA and protein expression should be examined within the same sample so that not only may the mechanism of overexpression be potentially assessed but a true picture of the functional unit ie. the protein, be ascertained.

Figure 4.3 Expression of the human pi GST subunit in the cytosolic fractions of leucocytes from controls and leukaemia patients.

Cell cytosols were prepared as described in section 2.2.1

25µg of soluble protein was subjected to SDS/PAGE, transferred to nitrocellulose and probed with specific antibodies raised against the human class pi subunit (π). Stds. = standards; N = nomal controls; T = leukaemia patients.



CHAPTER 5

THE ROLE OF THE GLUTATHIONE S-TRANSFERASES IN A STRESS RESPONSE

5.1.0 INTRODUCTION

The work described in Chapter 3 revealed that in a lung tumour cell line made resistant to 1-chloro-2,4-dinitrobenzene, both alpha and pi class GST subunits were overexpressed. Initially this cell line was derived in order to examine the role of the GST in the phenomenon of drug resistance but the elevated expression of alpha and pi class subunits did not appear to confer resistance to any of the antitumour agents analysed in this model (see Figures 3.23 to 3.26). The changes with respect to GST expression, that occurred in the $CDNB^r$ line did however afford protection against cytotoxic insult from cumene hydroperoxide Fig. 3.22).

As well as being implicated in certain drug resistance models, it has also been suggested that the GSTs may play a role in the stress response (Lewis,1988; Hayes & Wolf, 1990). This response describes the process whereby a cellular population can adapt to protect itself against a number of physiological stresses. These include heat shock, high oxygen concentrations, ultra-violet irradiation, alcohol, osmotic shock, pH changes and viral infections (Burdon, 1986; Lindquist, 1986; Pelham, 1986; Subjeck & Thung-Tai, 1988; Yalkinoglu et al, 1988).

Besides affording protection against the actual physiological insult endured, it has

been shown that the stress response may also result in protection against the toxic effects of certain drugs (Li and Hahn, 1978; Li et al, 1982; Li, 1987). The mechanisms whereby cellular populations protect themselves from certain physiological stresses are unclear.

The response to heat shock is the most well studied and understood stress response. During this process a transient arrest in cell growth has been noted which is accompanied by a block in the synthesis of DNA, RNA and protein. Concomitant with this arrest of macromolecule biosynthesis is an increase in transcription of a small number of genes that code for the heat shock proteins (hsps) (Welch et al, 1989). It is thought that expression of these hsps confer resistance to potentially lethal temperature increases.

The mechanisms by which the hsps may confer thermotolerance on cells is to a large extent unknown as are the exact functions of the majority of these proteins. It is thought that members of the hsp 70 family are involved in protein-protein interactions (Welch et al, 1989).

The hsps have been shown to interact with p53, which is a protein expressed in the nuclei of cancer cells. p53 has also been shown to be induced by carcinogens and oncogenes (Pinhasi-Kimhi et al, 1986). The hsp 70 proteins may also be involved in actin microfilament reassembly and also recovery of nuclear morphology following exposure to abnormally high temperatures (Pelham, 1986; Burdon, 1986).

Other proteins of higher and lower molecular weight than the hsp 70s have also been shown to be induced in response to certain stresses (Burdon, 1986). However, they remain poorly characterised.

The idea that the GSTs may in some way be involved in a stress response has arisen from several lines of evidence. Certain enzymes involved in the metabolism of glutathione have been implicated in the protection of prokaryotes from oxidative stress and glutathione itself has been postulated to participate in the induction of a stress protein (Christman et al, 1985; Shelton et al, 1986).

In the work that is described and discussed below, the expression of certain stress response proteins was analysed in the $CDNB^r$ line. The relationship between expression of stress response proteins and the sensitivity of the WT and $CDNB^r$ cell lines to elevated temperatures was also analysed.

5.2.0 RESULTS AND DISCUSSION

5.1.0 Expression of p53 in WT and CDNB^r cell lines

The expression of hsp 70 and the nuclear protein p53 in both WT and CDNB^r cell lines was analysed using the technique of immunohistochemistry. These analyses were carried out as detailed in the Materials and Methods Section 2.7.0

The results using monoclonal antibodies to different epitopes on p53 are shown in Figures 5.01 and 5.02. It is evident from these results that the expression of this protein is elevated in the CDNB^r line as judged by the intensity of immunohistochemical staining of the nuclei.

One notable feature in the nuclei of the CDNB^r cell line in Figures 5.01 and 5.02 is the apparent increase in size of the nuclei. The reason and implication of this is unknown. It is possible that DNA replication has occurred, but for some reason the nuclei have failed to divide.

The elevated expression of p53 in the nuclei of the CDNB^r cell line would be consistant with the report that hsp 70 can act to stabilise p53 (Pinhasi-Kimhi et al, 1986). It is possible that the observed elevation of p53 seen in the CDNB^r model is the result of hsp 70 mediated stabilisation of this protein since hsp 70 is also seen to be elevated in CDNB^r (See section 5.2.0).

It is possible that hsp 70 has the capacity to stabilise other proteins and that is one of the ways that cells expressing stress response proteins such as hsp 70 can resist abnormally high temperatures. It has recently been speculated that hsp functions are concerned with refolding and/or removal of denatured proteins when they are damaged by conditions such as elevated temperatures and pH changes (Welch et al, 1989).

Fig 5.01 Immunohistochemistry using a monoclonal antibody to p53 (Antibody 240, undiluted)

Immunohistochemistry was carried out on fixed cells as described in the Materials and Methods, section 2.7.0. The stained cells were photographed at a 200x magnification after the plates had been developed

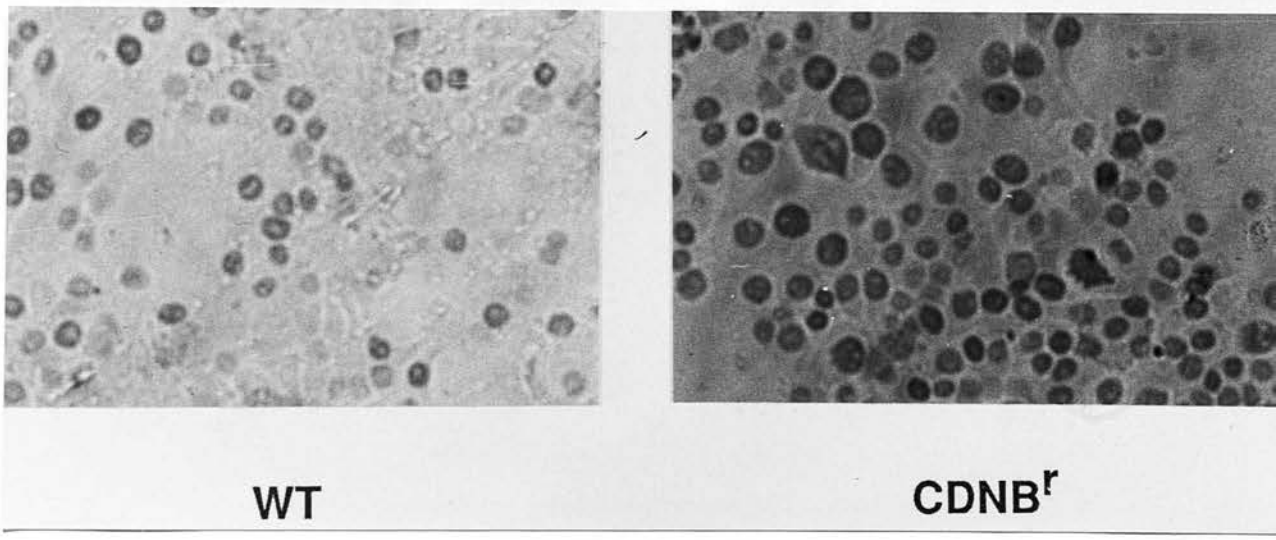
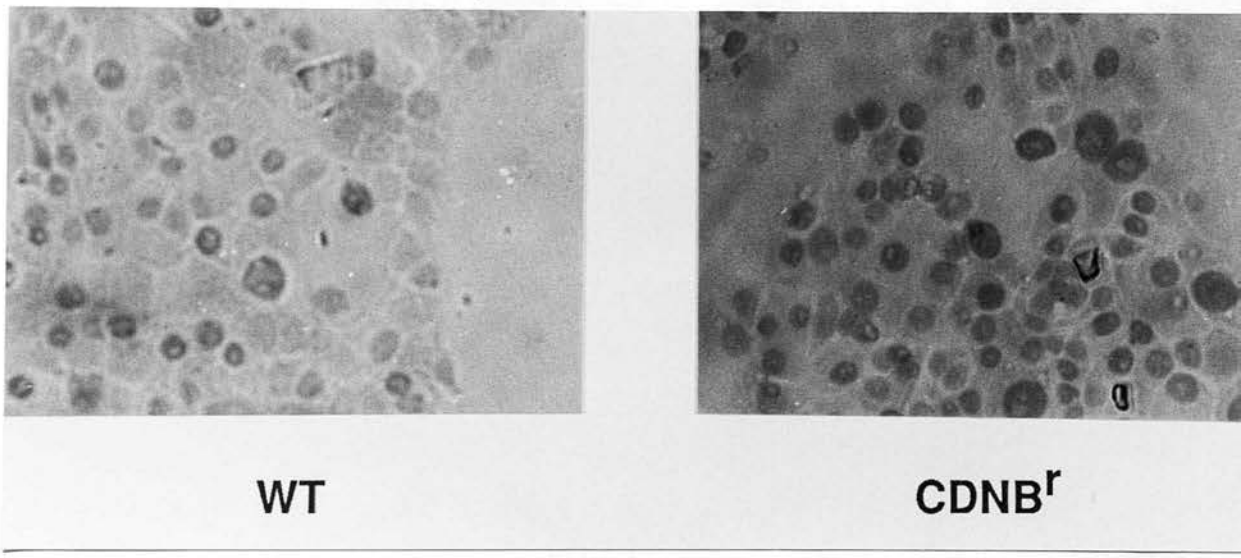


Fig 5.02 Immunohistochemistry using a monoclonal antibody to p53 (Antibody 1801, undiluted.)

Immunohistochemistry was carried out on fixed cells as described in the Materials and Methods, section 2.7.0. The stained cells were photographed at a 200x magnification after the plates had been developed



5.2.0 Expression of hsp 70 in WT and CDNBr cell lines

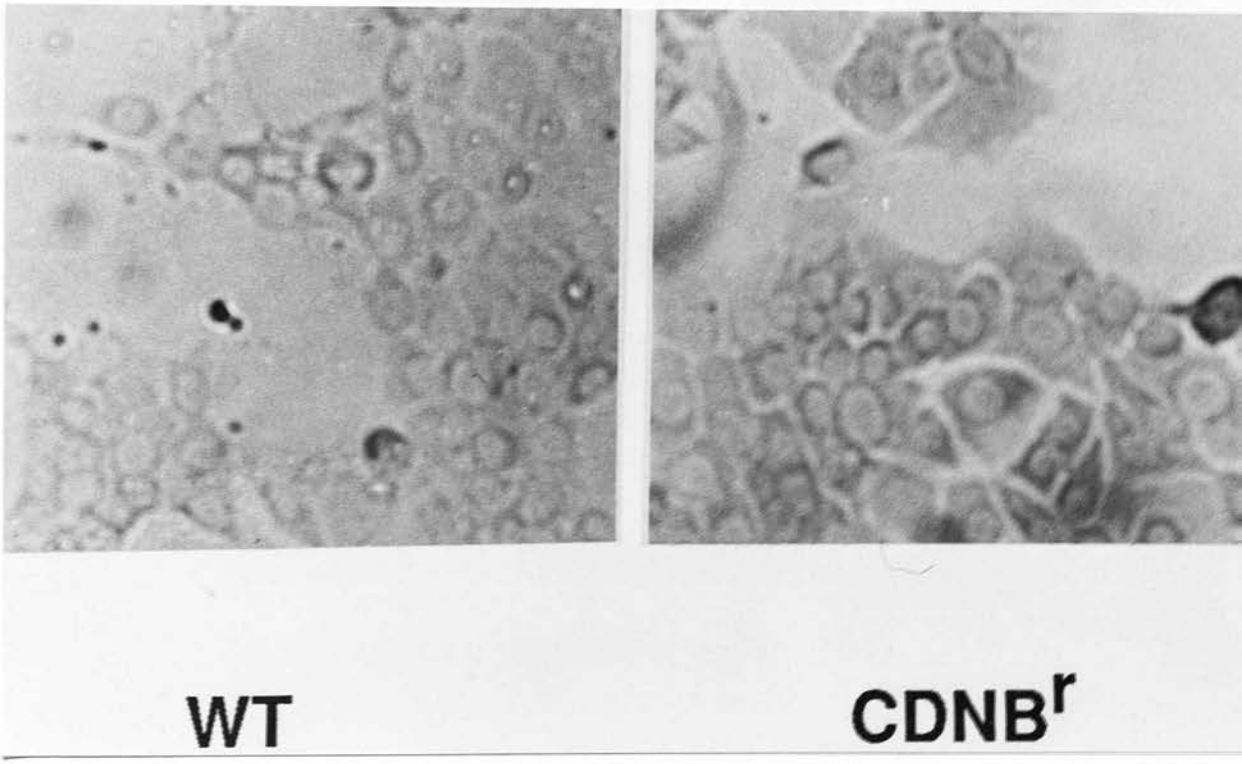
The results from the immunohistochemical analysis of the WT and CDNB^r cell lines using a monoclonal antibody to hsp 70 are shown in Figure 5.03. Hsp 70 also seems to be overexpressed in the CDNB^r line when compared to the WT. Hsp 70 expression as mentioned in the introduction to this chapter is switched on in response to a number of stresses including heat and high oxygen concentrations. From the information presented in Figure 5.03 it appears that 1-chloro,2,4-dinitrobenzene can also be added to the list of agents that induce heat shock proteins.

The family of proteins known as the heat shock proteins (they were first noted in cells after exposure to abnormally high temperatures) have received much attention in the last few years (Burdon, 1986; Lindquist, 1986; Pelham, 1986; Subjeck & Thung-Thai, 1986). Although their function and mode of action have been extensively researched, the emerging picture is complex. During heat shock in various organisms a number of proteins of varying molecular weight have been shown to be induced. These include proteins of molecular weight 110 kD, 95 kD, 84 kD, 70 kD and a range of smaller proteins of around 20-30 kD (Burdon, 1986). Of these proteins, hsp 70 has been shown to be the most readily detectable in most of the organisms studied to date. Using immunohistochemical techniques hsp 70 has been found at low levels in the cytoplasm of cells at normal growth temperatures but high levels were observed in the nucleus and nucleolus of cells after heat shock (Welch & Feranisco, 1984).

The fact that hsp 70 appears to be induced in the CDNB^r cell line raises some interesting questions. Since the hsp(s) are expressed in the CDNB resistant cell line, which is cultured in the absence of CDNB,they are actually expressed in the absence of any immediate stress, suggesting that their overexpression is not of a transient nature. It is possible that the gene(s) encoding the hsp 70 protein have not been "switched off" once CDNB has been removed or that some sort of mutational event during the

Fig 5.03 Immunohistochemistry using a monoclonal antibody to hsp 70.

Immunohistochemistry was carried out on fixed cells as described in the Materials and Methods, section 2.7.0. The stained cells were photographed at a 200x magnification after the plates had been developed.



derivation of the CDNB^r cell line has led to permanent expression of hsp 70. This may have been either a mutation in a regulatory sequence of the hsp 70 gene(s) or a mutation in gene sequences of factors that regulate hsp 70.

5.2.3 Thermotolerance of WT and CDNBr cell lines

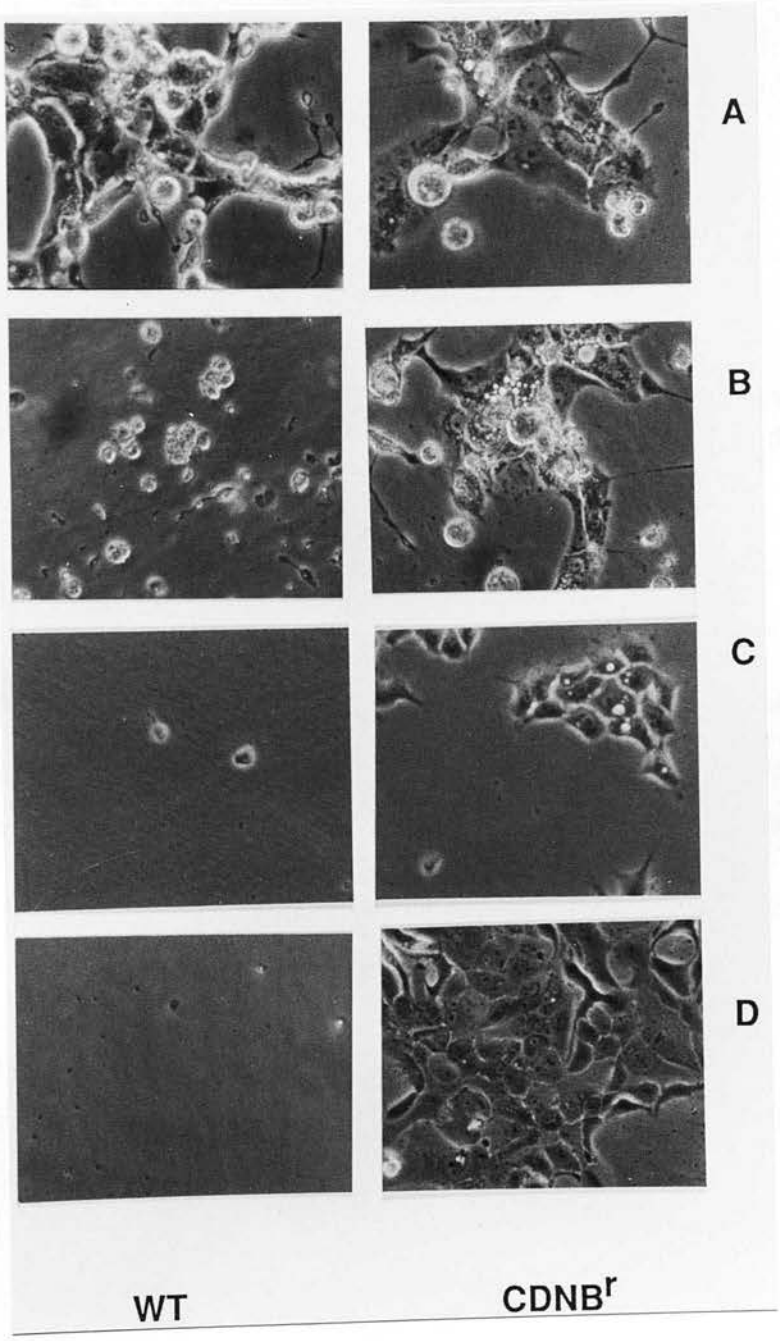
The CDNB^r cell line exhibited some degree of resistance to elevated temperatures (Figures 5.04 and 5.05). In Figure 5.04, the qualitative changes seen in both WT and CDNB^r cell lines when incubated at 42°C for 5 days and then returned to 37°C, are shown. The two cell lines were plated out at equal plating densities in order to rule out, as far as possible, the effects of different cell densities on survival rates. What seemed to occur is that the CDNB^r cells, although showing a certain amount of cell death during incubation at 42°C, appeared to recover when they were returned to 37°C. The WT cells on the other hand showed signs of cell death at 42°C as did the CDNB^r cells, but when the WT cells were returned to 37°C they did not recover and after 12 days at 37°C, no viable cells were observed.

In order to try and quantify this observation, WT and CDNB^r cell lines were plated out at equal cell densities in 96-well plates. They were then incubated at 42° for 40 hours and then returned to 37°C for a further 10 day incubation. MTT was then added and the plates processed as described in Section 2.1.6. The results are shown in Figure 5.05. The optical density readings were used to measure cell viability. It is evident that although there were some viable cells after heat shock for 40 hours, comparatively more CDNB^r cells were observed after heat treatment.

To summarise the results presented above, the model that has been generated during the derivation of the CDNB^r cell line is one which overexpresses both alpha and pi class GST and is resistant to both CDNB and cumene hydroperoxide. As well as the changes with regards to GST expression, the CDNB^r cell line also appears to overexpress hsp 70 and exhibits some degree of resistance to abnormally high temperatures. It is possible that

Fig 5.04 Survival of WT and CDNB^r cell lines after heat shock at 42^{o}C for 5 days followed by 12 days of culture at 37^{o}C

Cells were harvested as described in the Materials and Methods, section 2.1.5. WT and CDNB^r cells were plated out at equal cell densities in 75cm^2 culture flasks and allowed to attach for 12 hours. The cells were then transferred to an incubator at a temperature of 42^{o}C. Cells were incubated at this temperature for 5 days and were photographed daily during this period. The cells were then returned to 37^{o}C and left for 12 days during which time they were photographed on a daily basis. A = 3 days at 42^{o}C; B = 5 days at 42^{o}C, 1 day at 37^{o}C; C = 5 days at 42^{o}C, 7 days at 37^{o}C; D = 5 days at 42^{o}C, 12 days at 37^{o}C.



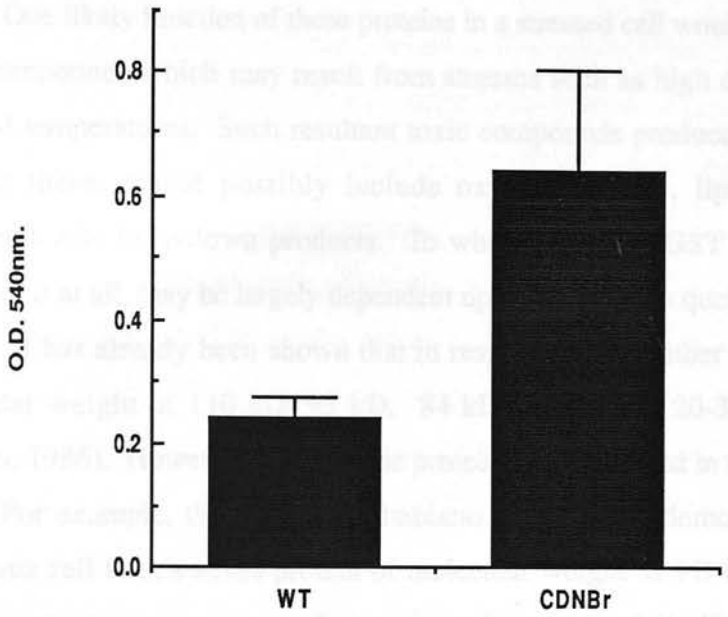


Fig. 5.05. Survival of WT and CDNB^r cell lines (as measured by O.D. at 540nm following treatment with MTT) after heat shock at 42^{o}C for 40 hour followed by 11 days of culture at 37^{o}C.

Cells were harvested as described in the Materials and Methods, section 2.1.5 WT and CDNB^r cell lines were plated out at equal cell densities in a 96-well culture plate and incubated for 40 hours at 42^{o}C followed by 11 days at 37^{o}C. MTT was then added to the plates and after 4 hours these were developed as described in the Materials and Methods (section 2.1.6). The optical density of each well was measured at 540nm.

certain GSTs can function as stress response proteins in that their expression is either "switched-on" or elevated in response to cellular injury.

One likely function of these proteins in a stressed cell would be the detoxification of toxic compounds which may result from stresses such as high oxygen concentrations or elevated temperatures. Such resultant toxic compounds produced in response to stresses such as these, would possibly include oxygen radicals, lipid peroxides and other macromolecule breakdown products. To what extent the GST are involved in a stress response, if at all, may be largely dependent upon the stress in question.

It has already been shown that in response to a number of stresses, proteins of a molecular weight of 110 kD, 95 kD, 84 kD, 75 kD and 20-35 kD may be expressed (Burdon, 1986). However, not all these proteins are expressed in response to all stresses.

For example, the work of Caltabiano et al (1986), demonstrates that in a human melanoma cell line, a stress protein of molecular weight 32 kD is induced in response to sodium arsenite, zinc, copper, cadmium, iodoacetamide and disulfiram, but is not induced in response to physical hyperthermia. Although not all stress response proteins are expressed in response to all stresses, hsp 70 does seem to be expressed in response to most stresses.

In prokaryotes it has been shown that certain glutathione metabolising enzymes may be involved in protection from oxidative stress. Christman et al (1985) showed that in *S-typhimurium* there is a coordinated expression of glutathione peroxidase, glutathione reductase as well as superoxide dismutase, catalase and at least 30 other proteins, in response to hydrogen peroxide and other oxidants such as cumene hydroperoxide and tetra-butyl hydroperoxide.

This work was extended to show that hydrogen peroxide inducible proteins in *S-typhimurium* overlap with certain proteins induced by heat shock, that is, 5 of the 30 or so proteins induced in this bacteria by hydrogen peroxide are also induced in response to elevated temperatures (Morgan et al, 1986). These workers also showed that other stresses such as nalidixic acid and ethanol also induced certain of the 30 or so proteins that were induced by hydrogen peroxide.

It may be that for each type of stress, a specific set of genes are induced that code for stress response proteins that protect against specific stresses. It appears from the above discussion however, that cellular responses to particular injurous agents are interrelated, as

cells appear to express overlapping sets of proteins when subjected to various stresses.

It is possible that in the $CDNB^r$ cell line, in response to cytotoxic insult with CDNB, a stress response has been elicited which involves both induction of known stress response proteins such as hsp 70 and induction/elevation of proteins that can specifically detoxify toxic injurous agents. Whether in the $CDNB^r$ model, the GST can also be classed as stress response proteins is not known.

An association between adaptation to stress and acquired resistance to drugs has already been established by several workers. It has been shown that the response provoked by a number of physiological stresses such as heat, anoxia, ethanol and oxidative stress can lead to protection against certain drugs. Li and Hahn (1978) established a link between thermotolerance and drug resistance when they showed that ethanol could induce resistance to both adriamycin and heat in Chinese hamster ovary cells. Li et al (1982) went on to show that other agents capable of inducing heat shock response such as hypoxia, arsenite and cadmium also resulted in resistance to adriamycin.

Subsequent to this work, thermotolerant cells have also been shown to exhibit resistance to other antitumour agents such as actinomycin D, bleomycin and VM26 (Wallner and Li, 1986; Li, 1987; Hahn et al, 1989). How agents that induce a heat shock response also lead to resistance to certain anticancer drugs is far from clear. It could be speculated that certain proteins are induced alongside stress response proteins that somehow specifically confer resistance to the drugs.

The expression of stress response proteins in drug resistant cells has rarely been investigated. It would be interesting to analyse the expression of stress response proteins in drug resistant cells since it is possible that cells challenged with cytotoxic drugs may elicit some sort of stress response. It has already been shown that the drugs adriamycin and VM26 can both induce hsp 70 transcription in *drosophilla* and Chinese hamster fibroblast cells (Rowe et al, 1986). There has also been a report that BCNU can induce hsp 70 RNA in certain cells (Schaefer et al, 1988).

It has been noted that certain thiol reactive agents, including CDNB induce a stress protein of 30 to 35 kD in rat fibroblast cells (Shelton et al, 1986). As mentioned earlier, induction of a 32 kDa stress protein by sodium arsenite, heavy metals and thiol reactive

agents in a human cell line has also been reported (Caltabiano et al, 1986). Concomitant induction of other stress proteins of molecular masses 100, 90 and 72 kDa was also observed.

Although the stress response proteins of molecular weight 70 kDa have received much attention in recent years and are by far the best characterised, the same cannot be said of the proteins in the 20-35 kDa range. Their identity, physiochemical properties and functions are largely unknown. However, it is interesting that using affinity chromatography, unidentified proteins ranging in size from 30-35 kDa have been shown to bind to S-hexyl glutathione columns (Hayes et al, 1987).

Once the identity of the proteins that are elevated in cells treated with CDNB (Shelton et al, 1986) sodium arsenite, heavy metals and thiol reactive agents (Caltabiano et al, 1986) has been established, comparisons with other proteins can be made. If these stress induced proteins are found to share similarities with the proteins that elute from S-hexyl glutathione colums (Hayes, et al, 1987), then it is likely that they are involved in glutathione metabolism in some way. The identity of the stress proteins identified by Shelton et al (1986) and Caltiabiano et al (1986), however, remains to be elucidated.

In conclusion, the relationship between GST and hsp 70 overexpression in the $CDNB^r$ model remains to be established. Also information regarding stress protein expression in cell lines resistant to anticancer drugs is eagerly awaited. As discussed earlier, it seems likely that induction of subsets of stress proteins may be dependent upon the stress in question, although there seems to be an overlap in the proteins expressed during differnt types of stress.

The implications of the apparent link between stress response and drug resistance are unknown. Stress response protein expression in drug resistant cells, (if indeed, this occurs) may be part of a general protection mechanism that is elicited in response to cellular damage. Other proteins that provide specific protection against toxic species may be coinduced with stress proteins and as such may also be regarded as stress response proteins. The GST may fall into this latter category.

CHAPTER 6

CONCLUSIONS

It has been shown during the course of the work presented above, that directed overexpression of alpha and pi class subunits can be achieved in cell lines made resistant to the cytotoxic GST substrates,ethacrynic acid and CDNB. In the CDNB resistant lung tumuor subline that was derived, resistance to cumene hydroperoxide was also shown. That these compounds are known to be metabolised by certain GST isoenzymes, is indicative that the elevated GST subunits are responsible, at least partially, for the development of resistance to CDNB and cumene hydroperoxide. It is likely that resistance to the two cytotoxic GST substates has resulted from increased GST catalysed conjugation of CDNB and cumene hydroperoxide to glutathione.That CDNB and cumene hydroperoxide differ in their structure and mechanism of action is in itself interesting since it shows that resistance to diverse cytotoxic compounds may occur via potentially one mechanism. In the work described above, it is proposed that this mechanism is increased drug deotoxification, catalysed by the GSTs.

There are many reports of increased GST activity in tumour cell lines resistant to a range of structurally and functionally unrelated anticancer drugs.(Table 1.5) and also data has been presented that certain anticancer drugs can act as GST substrates (Dulik et al, 1989; Smith et al, 1989). Further work has shown that when cDNAs encoding GST subunits are transfected into eukaryotic cells, they confer resistance to a number of anticancer drugs

(Black et al, 1990). The data described above, taken together with the results presented in this thesis, indicate that the GSTs may play an important role in drug resistance. The wide ranging substrate specificity of the GSTs mean they probably have the potential to detoxify and so confer resistance to, a number of different anticancer drugs. However, infomation regarding the ability of anticancer drugs to act as GST substrates is necessary, in order to substantiate this.

The sensitvity of the derived CDNB resistant lung tumour cell line to a number of antitumour agents was investigated but there were no observable cross resistance patterns to any of the compounds analysed. In the light of these data, the possible involvement of the GSTs in a stress response was studied since challenging cells with cytotoxic compounds may possibly be regarded as a physiological stress.The CDNB resistant cell line was found to be overexpressing a hsp70 and also exhibited a degree of tolerance to elevated temperatures. It was concluded from this work that GSTs may play a role in stress response by possibly detoxifying harmful products resulting from the stress in question. In the work presented in this thesis, the stress in question is cytotoxic insult.

GST pi expression was investigated in leukaemia samples from a number of treated and untreated patients.The former group had been treated with a variety of anticancer drugs and had either relapsed or were unresponsive to treatment. Like much of the work in this field, the results were ambiguous, with overexpression of GST pi seen in some unresponsive tumours but not in others. Of the 32 chronic lymphocytic leukaemias analysed, 21 out of 25 chlorambucil treated patients showed increases in expression of pi GST suggesting that in this group of patients, GST pi overexpression may be one of the ways that leukaemia cells overcome cytotoxic drug concentrations.

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